PLANT PHYSIOLOGY

VOLUME 31 SEPTEMBER, 1956 NUMBER 5

INFLUENCE OF LIGHT AND DARKNESS UPON CARBON DIOXIDE FIXATION[1,2]

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For plants sensitive to photoperiodism light is generally essential for flowering, and the requirement for CO_2 with the light indicates a photosynthetic function for the light period (21, 10). There appears to be an additional role of light in which it apparently alters a photoreceptor pigment controlling floral initiation (22).

Evidence has been advanced recently that CO_2 is also essential during the dark period for floral initiation in some short day plants (13) as also in the allied phenomenon of vernalization of Alaska peas (15). Gregory et al (8) and Langston and Leopold (13) have suggested that the CO_2 metabolism in darkness is closely related to the photoperiodic response of short day plants.

It would be interesting to know more concerning the products of CO_2 fixation in this connection. Calvin and co-workers (4) have demonstrated that products of dark and light fixation of CO_2 by green plants are dependent upon preceding light and dark conditions. It has long been accepted that some materials synthesized in a high intensity light process are utilized in the long dark period essential for flowering of short day plants (9). This suggests that a study of C^*O_2 fixation in light and darkness during the period of photoperiodic induction with the aid of chromatographic and autoradiographic techniques may yield useful information regarding the biochemistry of the photoperiodic reactions.

MATERIALS AND METHODS

Two short day species—Biloxi Soybean (*Glycine Max* L.) and Cocklebur (*Xanthium pensylvanicum* Wallr.), and one long day species—Wintex barley (*Hordeum vulgare* L.), were used. These were grown on gravel beds with nutrient solutions. Induction treatment was begun when the plants were at least 3 weeks old. Short and long day species were maintained in the vegetative condition by subjecting them to 16- and 8-hour photoperiods, respectively. For floral induction the respective photoperiods were 8 and 16 hours.

Carbon dioxide fixation was studied with detached leaves except in the case of barley (the bulk of which is leaves) when only the roots were removed. The leaves of soybean and cocklebur were selected to have a midrib length of 6 to 8 cm; for cocklebur such young leaves have been shown to have the maximum sensitivity (11). The leaves were cut under water and the petioles were then dipped in a complete nutrient solution. The method of application of CO_2 was as described previously (13). The concentration of CO_2 in the experimental atmospheres was 0.25 %, and the radioactivity was approximately 25 μc/l of air. Where comparisons of 3 species are made in the tables, all 3 were exposed to the C^*O_2 simultaneously in the same chamber.

In the experiments on photosynthesis, the photosynthetic chamber was immersed in a glass water bath to minimize temperature changes inside the chamber. The chamber was evacuated briefly with an air circulating pump before C^*O_2 was introduced. The C^*O_2 was generated previously in a closed weighing bottle fitted with stop-cocks and forming a part of the assembly, and was swept into the experimental chamber with natural air when the stopcocks were opened. The light on the leaf surfaces was ca 3000 fc in intensity and was supplied by a 150 w reflector flood lamp for 15 minutes in all experiments.

The plant material was weighed before introduction into the chamber, and immediately after cessation of the experiment was dropped into boiling 80 % ethanol and extracted on a water bath for 20 minutes. The alcoholic extract was decanted, and the tissues further crushed and extracted with fresh 80 % ethanol on a Waring Blendor for 5 minutes and the extract filtered. The combined filtrates and washings were concentrated under vacuum to a small volume, and then diluted with water to a volume such that 1 ml of the extract would correspond to 1 gm of fresh tissue. When heat was applied care was taken that the temperature did not exceed 75° C. The concentrated extract was centrifuged if necessary and 50 to 100 μl of the extract solution was applied at a corner of Whatman's No. 1 filter paper and chromatographed two-dimensionally. The solvents used were water-saturated phenol and *n*-butanolacetic acid-water in the ratio by volume of 4:1:1. The papers were then exposed to Kodak Noscreen x-ray film (17″ × 14″), the exposure time depending on the activity of the spots. In most cases a one month exposure was enough. The radioactive areas of the paper were marked carefully with a pencil and counts taken di-

[1] Received November 29, 1955.

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rectly on the paper (5, 33). The spots were then cut out, eluted with warm water and tests for identification performed. For compounds not distinctly separated or those with large spot-areas aliquots of eluate were run in the same solvent for a longer time or in a different solvent. Most of the compounds were identified by color reactions, by R_f values in different solvents, and by chromatography with authentic samples.

The 80 % alcohol-insoluble residue was washed thoroughly, dried in an oven at 90° C ground to a fine powder on a micro-mill and the radioactivity counted in saturation thickness. Dark fixation samples were counted with a windowless gas flow counter, and photosynthetic fixation samples with an end-window counter.

Experimental

Preliminary experiments on the rates of CO_2 fixation in darkness by leaves of soybean, cocklebur and barley indicated that there were changes in these rates during the night. One would expect such changes on the basis of the data of Langston and Leopold (13). An analysis of the amounts of radioactive CO_2 incorporated during three-hour intervals by leaves of cocklebur is shown in figure 1. These data are only for components soluble in 80 % ethanol. It can be seen that the highest rates of dark fixation into the soluble fractions occurred during the first six hours of the night, and subsequent fixation into solubles was at a noticeably smaller rate.

Separation of the soluble products of dark fixation in leaves of soybean, cocklebur and barley produces data of the sort shown in table I. A zero or omission from the table indicates that there was no darkening of the film at the point expected for that compound, and a plus indicates that there was darkening but a degree of radioactivity too small to exceed 10 % of the background counts. Examination of the table shows that the bulk of the fixed carbon is in the phosphorylated sugars and phosphoglyceric acid, these compounds constituting some 20 to 80 % of the total radioactivity. Smaller amounts are detected in the organic and amino acid fractions. Especially notable is the absence of any free sugars. An interesting compound indicated as #8 was found on the soybean chromatograms, especially from photo-induced leaves. This compound has an R_f of 0.39 in phenol-water, and 0.29 in butanol-acetic acid-water. Its identity is not known. While the relative activities incorporated into the different compounds show differences between vegetative and induced plants, it is not possible at present to point to any of these compounds as having particular significance in the inductive process. These data reported in the table are representative of similar analyses made after 1, 3 and 5 inductive cycles, and with dark exposures to C^*O_2 of 16 hours as well as 8 hours.

There is an interesting possibility that the products of dark fixation of CO_2 may be altered by the

Table I

Products of Dark Fixation of C^*O_2 by Vegetative and Induced Leaves

Products	Soybean		Cocklebur		Barley	
	Veg	Induced	Veg	Induced	Veg	Induced
	cpm/100 mg tissue after 8 hrs darkness					
Hexose monophosphate	539	109	78	34	832	632
Hexose diphosphate	532	+	32	20	424	217
"Sugar" phosphate	111	105	0	0	103	0
Phosphoglyceric acid	113	69	279	138	103	230
Citric acid	53	17	20	80	73	98
Malic "	0	196	0	160	29	66
Fumaric "	14	+	0	35	0	0
Succinic "	+	0	16	19	6	11
Glycolic "	90	0	0	29	0	0
Glutamic acid	67	47	117	89	25	87
Aspartic "	177	+	73	30	83	54
Glycine	12	23	0	25	0	17
Glutamine	7	0	0	0	6	16
Asparagine	36	0	+	34	0	0
Threonine	+	23	+	0	11	13
Valine	13	0	0	+	+	+
Tryptophan	10	0	0	+	0	0
Leucine	+	0	0	+	0	8
Alanine	5	0	+	0	0	0
"8"	+	106	0	0	0	0
Total activity	1,826	828	782	753	1,767	1,471

Five cycles in soybean and barley, 3 cycles in cocklebur.

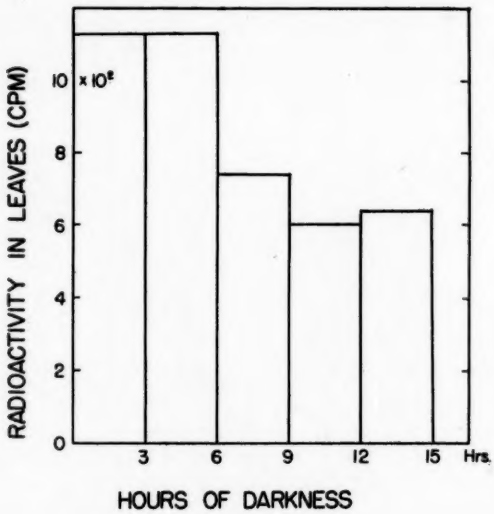

Fig. 1. The dark fixation of C^*O_2 into the ethanol soluble fraction of cocklebur leaves during a single long night. Separate groups of leaves taken from vegetative plants were exposed to C^*O_2 at successive 3-hr intervals during the night.

subsequent light period. In order to investigate this point, plants were exposed to C^*O_2 during the night period and then exposed to 15 min light in normal atmosphere before being plunged into boiling ethanol. Chromatographic separation of the tagged compounds showed no significant differences between the distribution of the radioactivity in those killed immediately after the dark exposure and those given subsequent light. The distributions in all three species were very similar to the data shown in table I.

Analysis of the products of photosynthetic fixation yielded the data shown in table II. Leaves of each of the three species were allowed to photosynthesize C^*O_2 for 15 minutes, and were then killed at once for analysis. In contrast to the situation after

TABLE II

PRODUCTS OF STEADY STATE PHOTOSYNTHESIS WITH C^*O_2 BY VEGETATIVE AND INDUCED LEAVES (5 CYCLES)

PRODUCTS	SOYBEAN		BARLEY	
	VEG	INDUCED 5 DAYS	VEG	INDUCED 5 DAYS
	cpm/100 mg tissue after 15 min light			
"Sugar" phosphate	17	0	0	0
Phosphoglyceric acid	47	110	67	2,610
Fructose	106	194	98	210
Sucrose	7,020	54,600	8,700	58,560
Glyceric acid	0	167	0	0
Citric "	205	378	18	381
Malic "	276	720	123	2,930
Fumaric "	52	34	0	62
Succinic "	100	181	+	96
Glycolic "	26	28	0	47
Glutamic acid	81	303	+	697
Aspartic "	47	64	0	67
Serine	70	1,382	334	131
Glycine	360	1,656	173	629
Phenylalanine	8	165	0	0
Glutamine	51	93	31	144
Methionine	28	303	0	0
Alanine	872	2,898	506	3,814
Total activity	10,014	66,014	10,329	69,771

dark fixation (table I), the bulk of the fixed carbon appears in the free sugar fractions. Fructose and sucrose together account for 70 to 90 % of the radioactivity in each soluble fraction. A surprising dearth of radioactivity in phosphorylated sugars was observed. Organic and amino acids were generously marked, with particularly high activity for alanine—a point that will be brought out further in a later experiment.

In an effort to determine whether the previous dark period might influence the photosynthetic rate of CO_2 fixation, an experiment was carried out with soybean, holding vegetative leaves in darkness for various lengths of time before exposing them all to 15 min of light in the presence of C^*O_2. As shown in figure 2, it was found that under these circumstances, the total radioactivity fixed was very much

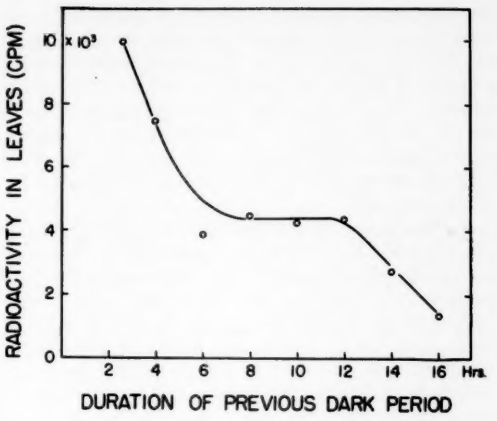

FIG. 2. The influence of the previous dark period upon the fixation of C^*O_2 in the light by Biloxi soybean leaves. Vegetative leaves were held in darkness for the times indicated, and then exposed to C^*O_2 in light for 15 min before total radioactivity in the tissues was determined.

lowered by previous extended periods of darkness, comparable in duration to long photoperiodic nights. A dark period of 6 hours or longer was followed by relatively retarded photosynthetic assimilation, and a period of 14 hours or more had even more pronounced slackening effects.

The inhibitory effect of one long dark period is further illustrated in figure 3, where the data for

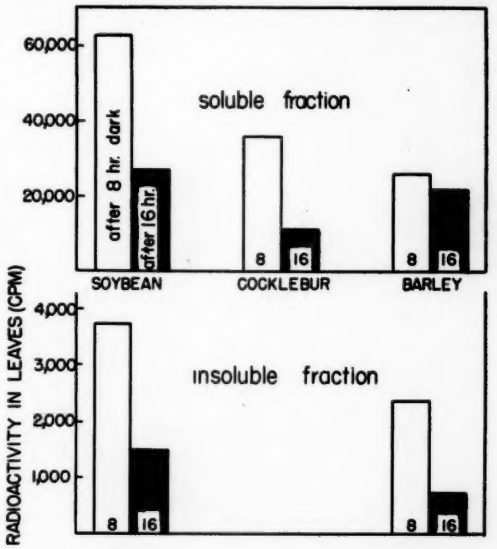

FIG. 3. The influence of the preceding dark period on 15 min photosynthesis. The radioactivity of the ethanol soluble or insoluble fractions are compared for short and long night pretreatments.

photosynthetic fixation of C^*O_2 are compared in all three species after a single night period of 8 or of 16 hrs. At the beginning of this experiment all plants were in a vegetative condition. Both the ethanol soluble and insoluble fractions were counted, and it can be seen that large inhibitions of C^*O_2 fixation were obtained after the long dark period. These inhibitions are evident in both the soluble and the insoluble fractions.

A final point of interest concerning CO_2 fixation appeared to be the nature of changes of photosynthates during the dark period subsequent to light exposure. In order to investigate this point, leaves of each of the three species were allowed to fix C^*O_2 in the light for 15 minutes, at which time the radioactive gas was swept away and the leaves were then darkened for 16 hrs. Samples were killed for analysis immediately after the light period, after 8 hrs of darkness, and after 16 hrs of darkness. Chromatographic separation of the tagged compounds revealed some large changes during the dark. First of all, there was a very rapid evanescence of the entire carbon pool. The loss of radioactivity in the ethanol soluble fraction was so rapid that 40 to 60 % of the radioactivity was lost in 16 hrs. The changes in the major groups of tagged compounds are shown in figure 4. It can be seen in the figure that the sugars—which constitute 70 to 90 % of the tagged soluble compounds—disappear at a rate very similar to the rate for total activity. A greater diversity of disappearance rates was found for the organic acids and amino acids. The former fell off in a manner parallel to the total activity in soybean leaves but they showed marked increases during the first 8 hrs of darkness in cocklebur and barley. These increases imply synthesis of organic acids from compounds produced during the immediately preceding light period—probably the sugars. The synthesis of amino acids might logically be expected to follow the organic acids, and in fact they showed simultaneous increases at 8 hrs in both soybean and cocklebur leaves. In barley the disappearance rate of amino acids was slow—considerably slower than the disappearance of activity in any other fraction. These data for the amino acids are plotted without including the data for alanine, because this compound experienced large and unique changes in the darkness. In all three species studied here, there were large amounts of radioactivity in alanine after light fixation periods, and in every instance this activity was quickly lost from the darkened leaves. A separate curve for alanine is plotted in figure 4 for each species. This compound at the beginning of the dark period showed the highest radioactivity of any compound in the leaves except sucrose, and in every instance it had practically vanished after 8 hrs darkness.

Discussion

Studies of the profound effects of light and darkness upon metabolism in this and previous studies have established that these environmental factors have complex effects upon respiration (2, 6, 12, 18, 28) and especially upon the fixation of carbon dioxide (2, 8, 13, 20). Cycles of light and dark which

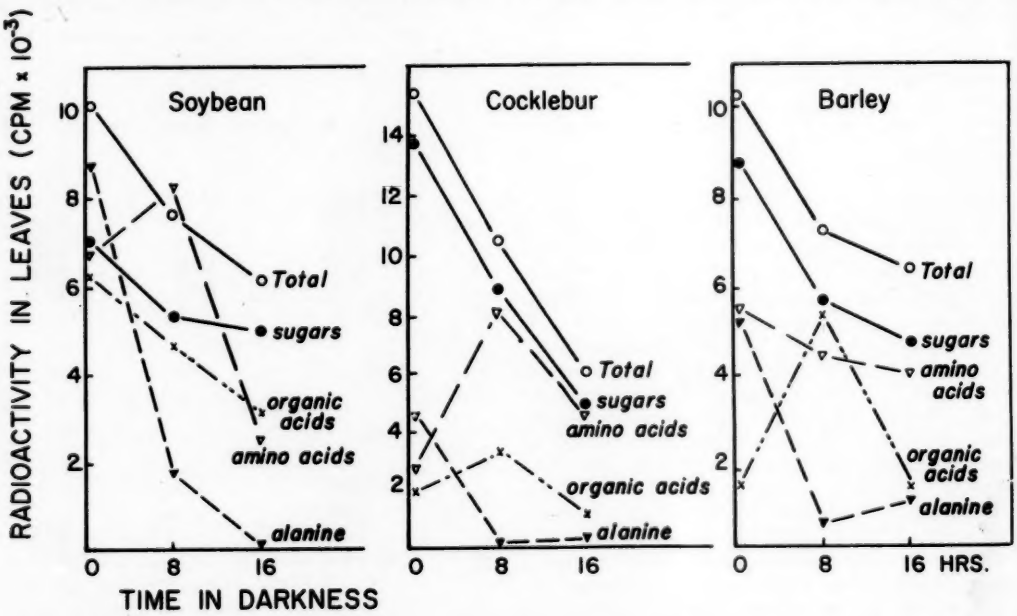

Fig. 4. Changes in the soluble products of photosynthesis during subsequent darkness. Values for organic acids, amino acids and alanine are all multiplied by 10 for clarity.

are known to have inductive effects upon plant reproduction alter markedly the fixation of CO_2 in darkness.

It was suggested some time ago by Bode (2) that photoperiodic induction might involve an increase in photosynthetic rates, though his experimental material was too far advanced in reproduction to permit precise comparisons. Similar studies by Norris and Calvin (20) are in agreement with Bode. The experiments reported here do not indicate such an increase with the first days of induction, but the length of the dark period does appear to influence photosynthetic rates. In each of the species tested, longer dark periods are correlated with lower photosynthetic rates during the light period (figs 2 and 3).

There are few published reports regarding the metabolic products of the dark fixation of CO_2 for long periods. C^*O_2 was used for comparatively long dark periods in some experiments involving sunflower (7), Kalanchoë (27), soybean (26) and barley (4). The photoperiodic circumstances of the plants used were not controlled, so the products obtained at the end of the dark periods do not assist in the understanding of the mechanism of photoperiodic induction. In only a few studies (23, 24) has attention been paid to the synthesis and utilization of individual compounds during extended night periods; in most instances in which they have been studied duration of the experiment was only a few minutes (3, 25) or a few hours (34).

In relation to the long established fact that a light period must precede a dark period for photoperiodic effectiveness (9), it is suggestive to note that sugars have been indicated as prerequisites for organic acid formation at night (23, 24, 29) and sugars are clearly not formed during the darkness. With respect to the products of dark fixation, it is interesting that malic acid—the major product of dark fixation in a wide variety of plant tissues (27)—is not detectable after 8 hrs in vegetative short-day plants, but is abundantly present in photoinduced plants (table I). In barley, relatively little organic acids are formed as a consequence of dark fixation, but very large amounts are formed in light (tables I and II). This species does not require a long night for flower initiation, nor does it require CO_2 in the atmosphere at night (13). Malic acid is particularly abundant after light fixation in barley, and this acid has been found to promote its flowering (14).

Among the amino acids, alanine provides most interesting data. This compound was found by Racusen and Aronoff (25) to accumulate quickly in the dark immediately following termination of light exposure, and it has also been shown to be formed photosynthetically probably via pyruvic acid (19). Whatever its means of synthesis, our data indicate that in prolonged dark periods, alanine is lost at the greatest rate of any compound measured.

One might conceive of light and dark as having four obvious possible interactions on the CO_2 fixation scheme: a) light may alter the fixation products of the previous dark period; b) it may alter the subsequent dark fixation, c) photosynthesis itself may be altered by the previous period of dark fixation; and d) the products of photosynthesis may be altered in the subsequent dark period. In this study we have examined each of these possibilities. As for the first, it was found that 15 min light had no perceptible effect upon the distribution of radioactivity fixed during the preceding darkness, and so it would appear that this possibility does not hold. However, each of the other three possibilities are supported by our data. The rate of dark fixation is decreased in time after termination of the light period (fig 1). Photosynthetic rates are strongly altered by the length of the previous dark period (figs 2 and 3). And the products of photosynthesis are rapidly and profoundly changed during the ensuing dark period (fig. 4).

For some time it has been held that light forms some precursor for a dark reaction (9), and this was the role of the light period in photoperiodism. Our data are entirely in agreement that the light period brings about the synthesis of some materials which will be altered in the dark, but they indicate that this may be only a part of the light effects. Sugar application has been found to replace effectively the light period in normal cocklebur plants (16), as well as in etiolated plants of long-day Raphanus (31) and of short-day Chenopodium (17) and Pharbitis (32). Gregory et al (8, 30) observed that light caused large bursts of CO_2 production in Kalanchoë plants which had been photoinduced, and Langston and Leopold (13) established that light interruption not only caused loss of much of the carbon fixed in the previous dark period but also retarded subsequent fixation in the remainder of the dark period for soybean and cocklebur. These findings along with the results of the present study, indicate that light brings about complex changes in CO_2 metabolism which may well be related to photoperiodism.

A problem in the interpretation of data of the sort reported here is the question of how much of the radioactivity in the leaves is due to exchange of CO_2 in equilibrium phenomena of the carboxylating enzymes, and how much is due to an actual net fixation or synthesis. The radioisotope technique as used here does not permit distinguishing between simple equilibrium phenomena and net fixation. Nevertheless, the experiments reported provide specific evidence as to the rates of the fixation phase of the CO_2 equilibrium reactions, and in addition they provide evidence as to the extent to which associated reactions alter the distribution of the fixed carbon. It is clear that these phenomena are strikingly altered by light and darkness, and many of the characteristics of these alterations suggest a relationship to photoperiodism.

Summary

A study of the light and dark fixation of CO_2 as it may be related to photoperiodism was carried out using two short-day species (Biloxi soybean and

cocklebur) and one long-day species (Wintex barley). Plants in precisely controlled states of photoperiodic induction were exposed to atmospheres containing CO_2 marked with C^{14}, and after fixation had occurred the leaves were killed and extracted in ethanol and the soluble materials into which the carbon had entered were identified by paper chromatography and autoradiography. The following conclusions were drawn:

1. The light period has an influence on dark fixation of CO_2. The longer the interval after light cessation, the slower the dark fixation by cocklebur leaves.

2. The dark period influences photosynthetic fixation of CO_2. The longer the preceding dark period, the lower the photosynthetic rate regardless of the photoperiodic requirement of the species. In addition, light fixation products were found to be rapidly changed in the dark. In particular, there was a rapid depletion of sugars, and usually a rise in organic and amino acids in the course of the night. A very rapid depletion of radioactivity in alanine was found.

3. It is concluded that there are compounded interactions between the light and the dark periods in photoperiodism, and that these interactions are reflected in altered patterns of CO_2 fixation both in light and in darkness.

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ATMOSPHERIC FLUORIDE: ITS UPTAKE AND DISTRIBUTION IN TOMATO AND CORN PLANTS[1,2]

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It has definitely been established in the past decade that plants can be adversely affected by high concentrations of fluoride in the atmosphere as well as by high concentrations of soluble fluoride in the soil. There now exists a fairly extensive bibliography concerning sources of fluorine contamination, injury patterns on plant foliage, and species sensitivity to fluorine compounds (1, 2, 3, 5, 6, 7, 8, 9, 10, 13). This paper deals with experiments designed to trace the fate of fluoride in growing plants as a result of exposure to a constant total amount of hydrogen fluoride gas administered at various time-concentration levels.

METHODS

Soil-grown tomato and corn plants were used in this experiment. The tomato series (variety Rutgers) was represented only by the blossom stage; whereas the corn series (variety U.S. 13) included two stages of growth, the early tassel and ear stages.

The fumigation apparatus consisted of a glass enclosed chamber, $4' \times 8' \times 6'$, which provided for a complete air exchange once every minute. Fluorine was injected into the air stream of the chamber from a thermostatically controlled wooden structure where it was evolved by dripping paraffin oil from a funnel in which the oil level was held constant, through a calibrated capillary tube into a polyethylene container filled with an air-hydrogen fluoride mixture. The displaced fluorine was passed through polyethylene tubing into a flue where it was uniformly mixed with incoming water-scrubbed air. A series of baffles provided for uniform mixing and distribution of the gas mixture throughout the chamber. The concentration of fluoride in the chamber was determined by continuously withdrawing an air sample at a height of 5′ through a series of scrubbers containing distilled water and a few drops of isopropyl alcohol to facilitate scrubbing. The resulting solution was titrated according to the standard procedure for fluoride determination (12). The concentration of hydrogen fluoride could be changed by varying the bore or length of the capillary tube, the speed of air exchange in the chamber, or the original air-hydrogen fluoride mixture. In these experiments, concentrations ranging logarithmically from 0.01 ppm to 1.0 ppm fluoride were desired. Actual concentrations evolved were 0.005, 0.10, and 0.65 ppm. All fumigations were continuous and were carried out at temperatures of 25 to 30° C and relative humidity of 56 to 60 %.

The experimental plan is indicated in table I. Twelve plants of each species were fumigated under each set of conditions, hereafter referred to as long-low, intermediate, and short-high fumigations. In every fumigation, the entire exposure was divided into three equal periods and a group of plants was removed from the chamber at the end of each period. Half of each group was placed in the greenhouse for further observation. The remaining half was harvested immediately and leaf samples were taken in the following manner. Tomatoes were separated into mature and young leaves and a portion of leaves of each age was washed for two minutes in agitated distilled water, the other portion remaining unwashed. At the end of one week the plants previously set aside were harvested in the same manner. Corn plants fumigated at the early tassel stage were separated into young and mature leaves. Those fumigated at the ear-bearing stage, having all mature leaves, were not separated. All corn leaves were fractionated into

TABLE I

EXPERIMENTAL PLAN FOR FUMIGATION OF TOMATO AND CORN PLANTS

DESIGNATION	TIME	F CONC	TOTAL F CIRCULATED
	hrs	*ppm*	*kgm*
Long-low	513	0.005	0.0011
Intermediate	26	0.10	0.0011
Short-high	4.25	0.65	0.0012

[1] Received January 31, 1956.
[2] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey.

tip, middle, and basal portions. Each portion was divided lengthwise along the mid-vein and one-half of each was washed and the other half left unwashed. At the end of one week the remaining corn plants were harvested in the same manner. In the case of the long-low fumigation, terminal growth was marked at the start of the fumigation and subsequent new growth was discarded before harvesting. A series of untreated control plants was also grown and similarly harvested. All leaf samples were dried to a constant weight in a forced-draft oven at 45° C and ground through the 40-mesh screen in a Wiley Mill, after recording dry weights.

Results and Discussion

Observations on Injury: Observations of the plants a week after fumigation revealed that the short-high fumigation had the most damaging effect on both tomato and corn plants. In the long-low fumigation, tomato showed no injury as the result of the first period and only very slight tip injury on the youngest fully-matured leaves as a result of the entire fumigation. The first period of the intermediate fumigation caused slight tip burn of the terminal leaves which increased slightly during the second and third periods. In the short-high fumigation there was slight to moderate marginal as well as tip burn of the leaves which became more severe until by the end of the third period there was injury not only to the younger growth but also to the mature leaves. In the latter case, necrosis was not confined to the margins but occurred within the leaf blade as well. This type of injury which has been observed on peach, cherry, prune, buckwheat, spinach, sorrel, and many other species seems characteristic of dicotyledonous plants (1, 2, 3, 6, 7, 10, 13).

Corn, in general, was more sensitive to fluorine than tomato. Symptoms developed more rapidly, and the injured area was more extensive. Corn fumigated at the tassel stage was even more sensitive than that fumigated at the ear stage. Similarly, as with tomatoes, the greatest injury in corn was derived from the short-high fumigation. In all three periods of the long-low fumigation the resulting injury was characterized by mottling of the mature foliage with only slight tip and marginal burn of the younger tissue. Mottling in corn has also been reported by Thomas (10) as a result of "fumigations insufficient to cause immediate collapse of the tissues." During the intermediate fumigation there was less mottling and a tendency toward longitudinal necrosis within the blade as well as tip or marginal burn. The short-high fumigation resulted in injury which was characterized by longitudinal necrosis or streaking on leaves of all ages and absence of mottling, again corroborating observations of Thomas wherein "at high concentrations such acute markings developed" (10). This type of injury appears most commonly on monocots such as wheat, oats, barley, rye, lily, and gladiolus (1, 2, 3, 5, 10, 13). In regard to expression of visible injury, hydrogen fluoride was found to parallel sulfur dioxide in that the degree of injury produced by a given total amount of the gas increased with the rate of application (11).

Analyses: Fluoride determinations were made according to a modification of the method of Willard and Winter (12). All fluoride values represent actual titration figures minus the content obtained for controls. Results are presented in tables II and III. Table II indicates that when tomato plants were exposed to a given total amount of fluoride (0.0011 mg F), the greatest total accumulation of fluoride as well as the most severe injury resulted from the short-high fumigation of 0.65 ppm for 4.25 hours. After the third period of this fumigation the leaf content (weighted average for young and mature leaves) was 359 ppm F or 1.4 times that of the leaves exposed to the intermediate fumigation. At the third period of the intermediate fumigation of 0.10 ppm F for 26 hours the fluoride accumulation in the leaf was 248 ppm or 1.2 times as much as in the long-low fumigation. At the end of the long-low fumigation of 0.005 ppm F for 513 hours, tomato foliage contained the lowest amount of fluoride, an average of 205 ppm, and showed the least damage. Results indicate that the amount of fluoride found in plants exposed to a given quantity of hydrogen fluoride increases with the rate of application.

Although two stages of corn plants were used in the experiment, values are given only for the tassel stage since data for the older series corroborated each principle. As was the case with tomato, when corn was exposed to a given total amount of hydrogen fluoride (table III) the highest fluoride accumulation

Table II

Fluoride Content* of Mature and Young Tomato Leaves Exposed to Varying HF Fumigations and Post-Harvest Treatments

Period	Post-harvest treatment**	Type of fumigation: Long-low, Mature	Long-low, Young	Intermediate, Mature	Intermediate, Young	Short-high, Mature	Short-high, Young
		ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
1	A	96	60	82	52	144	236
	B	30	14	48	32	78	150
	C	46	30	46	54	78	130
	D	20	14	46	30	76	88
1, 2	A	166	126	182	172	244	295
	B	86	48	124	150	149	195
	C	96	50	170	180	326	236
	D	50	34	132	116	236	200
1, 2, 3	A	256	140	264	210	344	340
	B	136	88	172	172	236	266
	C	131	90	260	198	416	410
	D	61	48	140	164	268	229

* Ppm on a dry weight basis.

** A. No treatment. B. Washing immediately. C. Standing for 1 week. D. Washing after 1 week.

and the greatest degree of injury resulted from the short-high fumigation and diminished in the intermediate and long-low fumigations. Calculations from data in table III indicate that at the end of the third period of each fumigation the total fluoride accumulation in corn leaves (weighted average for the entire leaf) was only half that of tomato leaves; 125 ppm F in corn as compared with 359 ppm F in tomato as a result of the short-high fumigation, 116 ppm F in corn as compared with 248 ppm F in tomato as a result of the intermediate fumigation, and 99 ppm F in corn as compared with 205 ppm F in tomato following the long-low fumigation. Leaves of corn plants exposed at the ear stage accumulated greater amounts of fluoride than did those at the tassel stage, but still less than did tomato leaves. The difference in fluoride content in the two species as well as the pattern of injury may be explained partially on the basis of anatomical features characteristic of monocots and dicots (4).

In order to illustrate the effect of leaf age of fluoride accumulation in tomato and corn, the ratio of fluoride in older tissues to that in younger tissues was calculated from the original data by determining the total leaf content and obtaining weighted averages. These ratios indicate that the more mature tomato leaves accumulated more fluoride than did the younger leaves during all periods of the long-low fumigation, the average ratio for all treatments being 1.5. This corresponds to the distribution gradient found in treating plants through the roots with sodium fluoride, where the mature leaves always accumulated more fluoride than did the younger leaves (1). During the intermediate fumigation, young and mature leaves accumulated almost equal amounts of fluoride, giving an average ratio of 1.0. In the short-high fumigation, on the other hand, the trend observed in the long-low fumigation was reversed in that young leaves generally accumulated more fluoride than mature leaves, the average ratio in this case being 0.8. In attempting to explain the high ratio in the case of the long-low fumigation, dilution by growth cannot be entirely ruled out, but numerous calculations from previous experiments (1) have indicated that such dilution is not solely responsible for the low fluoride content of the younger leaves. Thus is was concluded that as the fumigation level was increased the ratio of the fluoride content of the mature leaves to the fluoride content of the young leaves was reduced.

With respect to leaf age, unlike tomato, the mature corn leaves always showed a higher fluoride content than did the younger leaves. The calculated ratio of fluoride of the old/young leaves tended to be lower (1.3 in both the intermediate and short-high) than in the long-low fumigation where it was 2.3. Two exceedingly high ratios at the beginning of the long-low fumigation were not included in the average.

In comparing different portions of a particular corn leaf, fluoride accumulation was always greatest at the tip and decreased with distance away from the tip, both in the tassel stage and in the more mature plants. Since new growth is initiated at the base of a corn leaf, this is a further expression of the principle that fluoride uptake tends to be more pronounced in the old than in young tissues.

The question frequently arises as to the fate of fluoride after it has been taken up by the plant. It is conceivable that a certain amount may be absorbed in gaseous form externally on the leaf, or internally on the surface films of water bordering intercellular

TABLE III

FLUORIDE CONTENT * OF TIP, MID, AND BASAL SECTIONS OF MATURE AND YOUNG CORN LEAVES EXPOSED TO VARYING HF FUMIGATIONS AND POST-HARVEST TREATMENTS

PERIOD	POST-HARVEST TREATMENT **	TYPE OF FUMIGATION																	
		LONG-LOW						INTERMEDIATE						SHORT-HIGH					
		MATURE			YOUNG			MATURE			YOUNG			MATURE			YOUNG		
		T	M	B	T	M	B	T	M	B	T	M	B	T	M	B	T	M	B
		ppm	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*	*ppm*
1	A	61	36	20	34	12	20	73	57	56	70	42	46	83	61	52	68	42	34
	B	27	30	8	12	8	8	59	45	37	54	37	24	17	25	24	12	24	28
	C	43	30	12	6	4	2	42	40	20	49	23	23	62	68	30	62	48	20
	D	26	21	8	2	0	0	34	20	16	29	20	21	27	29	21	22	23	14
1,2	A	102	78	53	67	36	40	123	84	79	93	75	55	121	92	66	130	74	64
	B	86	48	32	34	30	22	96	73	65	73	59	41	78	75	31	74	59	38
	C	66	60	35	48	26	27	70	52	50	75	57	40	126	90	70	103	80	47
	D	65	44	18	17	15	12	65	44	37	62	34	30	68	54	46	58	49	44
1,2,3	A	121	100	130	110	58	51	129	125	114	107	110	80	177	140	101	143	130	75
	B	103	60	79	59	43	32	121	114	79	91	80	54	122	123	56	117	104	68
	C	76	84	90	70	41	38	84	87	82	86	78	72	193	157	118	149	116	69
	D	74	70	43	23	30	24	80	76	74	62	62	60	88	75	74	94	62	58

* Ppm on a dry weight basis.
** A. No treatment. B. Washing immediately. C. Standing for 1 week. D. Washing after 1 week.

spaces; or it may exist in other forms within the plant tissues combined with organic or inorganic cell constituents. To gain some idea of the disposition of fluoride in plants, various post-harvest treatments were applied to tomato and corn foliage. Table IV indicates the retentive capacity, or the percentage of total fluoride accumulation (treatment A) which was retained against post-harvest treatments B, C, and D by tomato and corn leaves during each fumigation period. Each value represents the weighted average for leaf tissue of entire plants. In both tomato and corn there was a trend toward greater fluoride retention with increased fumigation level. At the end of the long low fumigation both species showed the lowest retention averages, 46 % for tomato and 55 % for corn, as against 78 % and 72 %, respectively, after the intermediate fumigation, and 81 % for both species at the end of the short-high fumigation. With regard to post-harvest treatments, allowing plants to stand for one week following fumigation resulted in a greater fluoride retention than did subjecting the leaves to immediate washing. This was most pronounced at the late stages of the higher level fumigations where very little fluoride was lost by diffusion into the air. Washing leaves of plants a week following a fumigation lowered the percentage of fluoride retained to its lowest value.

Final consideration concerns the rate at which fluoride was accumulated and retained during the course of a fumigation. To this end percentage increment resulting from treatment A and from treatment D within successive periods of each fumigation was calculated and tabulated in table V. The rate of accumulation as well as retention generally tended to be constant during the course of fumigation. There was evidence in tomato, however, that in the last period of fumigation the rate decreased, indicating some degree of saturation had been reached.

From a practical standpoint certain principles have been illustrated by these experiments which may be applicable in interpreting observations and analytical data from the field. First, the ratio between the fluoride content of the mature and young leaves might give an indication of fluoride content in the air, since these experiments have shown that mature leaves were much higher in fluoride than young leaves after a low fumigation and the reverse was true after a high fumigation. The very fact of fluorine loss by washing or diffusion might serve as a means of distinguishing between gaseous air-borne and root-supplied fluorine in plants, since earlier work seems to indicate that there is no comparable loss in fluoride from mature leaves of plants treated through the roots with sodium fluoride as there is in plants fumigated with gaseous fluorine compounds (1). The post-harvest treatments also illustrate how, in addition to the time factor, some agencies such as wind and rain may operate under field conditions in modifying data derived from fluoride analyses of contaminated vegetation. In the case of corn, the presence of mottling may be a valuable clue in determining the conditions of a field fumigation since mottling appears

TABLE IV

PERCENTAGE OF FLUORIDE RETAINED BY TOMATO AND CORN LEAVES FOLLOWING POST-HARVEST TREATMENTS

PERIOD	POST-HARVEST TREATMENTS *	TYPE OF FUMIGATION: LONG-LOW TOMATO	LONG-LOW CORN	INTERMEDIATE TOMATO	INTERMEDIATE CORN	SHORT-HIGH TOMATO	SHORT-HIGH CORN
		%	%	%	%	%	%
1	B	28	60	60	72	60	47
	C	50	60	76	52	55	92
	D	23	36	57	39	43	47
	Av	34	52	64	54	53	62
1,2	B	46	65	78	81	64	65
	C	50	43	99	69	100	94
	D	28	44	69	51	80	62
	Av	41	51	82	67	81	74
1,2,3	B	57	63	73	80	73	76
	C	56	56	96	72	100	100
	D	25	46	65	64	71	66
	Av	46	55	78	72	81	81

* B. Washing immediately. C. Standing for 1 week. D. Washing after 1 week.

TABLE V

PERCENTAGE OF TOTAL AND RETAINED FLUORIDE IN TOMATO AND CORN * LEAVES FOR SUCCESSIVE PERIODS OF EACH FUMIGATION

PERIOD	SPECIES	TYPE OF FUMIGATION: LONG-LOW MATURE	LONG-LOW YOUNG	INTERMEDIATE MATURE	INTERMEDIATE YOUNG	SHORT-HIGH MATURE	SHORT-HIGH YOUNG
		%	%	%	%	%	%
1	Tomato						
	Total F	38	43	31	25	42	69
	Retained F	33	28	32	18	28	40
	Corn						
	Total F	24	25	49	53	41	41
	Retained F	26	0	26	36	32	29
1,2	Tomato						
	Total F	27	47	38	58	29	18
	Retained F	49	42	61	52	59	50
	Corn						
	Total F	33	42	23	19	25	33
	Retained F	35	54	30	24	35	44
1,2,3	Tomato						
	Total F	35	10	31	18	29	13
	Retained F	18	30	7	30	13	10
	Corn						
	Total F	42	33	27	28	34	26
	Retained F	39	46	44	40	33	27

* Corn figures represent weighted averages for tip, middle, and base fractions.

to be so characteristic of a long low-level fluorine fumigation.

Summary and Conclusions

Soil grown tomato and corn plants were subjected to a given total amount of fluoride as HF, administered at varied time-concentration levels designated as long-low, intermediate, and short-high fumigations, with the purpose of tracing the fate of the fluoride accumulated within the plants.

1. In both tomato and corn injury appeared more severe when the fluoride gas was administered in a short-high concentration dosage than when this same amount of gas was administered over a more protracted period. Corn was the more sensitive of the two species.

2. In both species there was an increased fluoride accumulation as the fumigation level was increased. Tomato has a fluoride-accumulating capacity approximately twice that of corn under all circumstances.

3. Mature leaves of both species accumulated greater quantities of fluoride than did younger leaves as a result of the more protracted fumigaions. At the shorter higher level fumigations the ratio of fluoride in mature to that in young corn leaves decreased to a certain point and then leveled off, whereas in tomato the ratio continued to decrease as fluoride concentration was increased. This ratio of old to young persisted whether applied to mature and young portions of a leaf (as in corn), mature and young leaves on a single plant, or mature and young plants of a single species.

4. These experiments corroborated earlier findings that a certain amount of absorbed gaseous fluorine is dissipated from both tomato and corn foliage during the short period of time after a long-low fumigation. Washing the foliage removed even more fluoride than did natural diffusion.

5. Fluoride retention in both species was greater as the result of a short-high fumigation than of longer and lower levels. The rate at which fluoride was absorbed and retained during the course of a fumigation was more or less uniform in the case of corn. In tomato there was a tendency for the rate to slow down in the late stage of the fumigation.

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THE BIOSYNTHESIS OF INDOLEACETIC ACID IN THE STYLES AND OVARIES OF TOBACCO PRELIMINARY TO THE SETTING OF FRUIT[1,2,3]

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The hormone responsible for the swelling of the ovaries of tobacco has been identified as indoleacetic acid (5). Indoleacetic acid (IAA) is probably the active principle responsible for the growth of strawberry receptacles (14). The biosynthesis of this compound has been studied and demonstrated in many plants and plant parts (3). Wildman, Ferri, and Bonner (21) were the first to suggest tryptophan as a precursor of IAA in higher plants. Prior to this, Thimann (18) had ascertained tryptophan to be an active precursor of IAA in *Rhizopus suinus*. The biochemical mechanism of this oxidation is not clear for all plants, but it is fairly well established that tryptophan is a precursor of IAA.

Free tryptophan has been studied in pollen of tobacco, acorn squash, and *Lilium regale* (13). Its occurrence has been reported in the ovaries of the same plants (13) and in the achenes of strawberries (14). The increase in tryptophan correlates nicely with previously found changes in IAA production (11).

There is a strong correlation between hormone synthesis in the styles of tobacco and the rate of pollen tube growth (5, 6). Muir (7) was the first to study hormone changes of pollen tubes grown in vitro on pure agar and sugar. Nitsch (12) found substantial increases in hormone after germination of pollen 13.5 and 20.5 hours on such a medium containing tryptophan. These experiments suggest that pollen tubes possess an enzyme system capable of converting tryptophan to IAA. These results were in question, however, because of possible bacterial contamination interfering with the oxidative process (13).

The possibility that the factor supplied by the pollen which brings about hormone production is an enzyme has been suggested (7, 19). Homogenates of resting pollen and unpollinated styles of *Lilium regale* contain a tryptophan-IAA enzyme (13). This enzyme activity increases with time after pollination in the ovaries of *Lilium regale* (13) and corn (17). However, doubt was cast on the possible participation of the enzyme in fruit development (8), because of the observation that both unfertilized and fertilized ovaries of Nicotiana contain an enzyme system which can convert tryptophan to auxin.

Therefore, the question which remains is, why is not auxin produced in the styles and ovaries of unpollinated flowers, if free tryptophan is present and tryptophan-IAA converting enzymes are present? This study was conducted in order to correlate the tryptophan changes in the styles and ovaries of tobacco with the hormone changes (5) after pollination. The enzyme systems of pollen, pollen tubes and pistils from unpollinated and pollinated flowers of tobacco were also studied to test the hypothesis that subsequent to pollination the pollen tubes induce the formation of a greater quantity of enzymes which are responsible for the synthesis of an additional amount of hormone, and hence bringing about development of fruits.

MATERIALS AND METHODS

Maryland Mammoth and Dixie Bright 102 were the two varieties of tobacco that were used in this study. Hand pollinations, as described earlier (5), were made at various time intervals in order to obtain pistils at different stages of development—during pollen tube growth, fertilization and ovary enlargement. Styles and ovaries were lyophilized and ground to pass a 40-mesh screen. This material was then used for tryptophan analyses.

TRYPTOPHAN ANALYSIS: The free tryptophan content of 40- to 50-mg samples was analyzed quantitatively using the *Lactobacillus arabinosus* assay as modified by Nitsch and Wetmore (15). Total tryptophan was determined by alkaline hydrolysis of 50 mg of lyophilized material in 10 ml of 4 *N* NaOH and an excess of cysteine to prevent oxidation. This process was conducted in an autoclave at 15 lbs pressure for 4 hours. After neutralization of the solution to pH 6.8 with HCl the extract was analyzed by the *Lactobacillus arabinosus* method (1). All samples were extracted with ether to remove anthranilic acid prior to analysis.

IN VITRO STUDIES WITH POLLEN: It was found that pollen of tobacco grows very well in 10 % sucrose and a mineral supplement of Larsen and Tung (4). Fifty milligrams of pollen were placed in 50 ml of such a solution on a rotary shaker operating at 180 cycles per minute. With this method the pollen and pollen tubes could be handled easily and quantitatively removed by centrifugation when used in enzyme preparations. Changes in hormone content of pollen tubes were followed in such a medium with and without tryptophan in order to follow the work of Muir (7) and Nitsch (12) with a different incubation system. Only 10 to 12 hours were used as a

[1] Received February 2, 1956.

[2] This paper constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University of Michigan in 1955. The work was made possible by an F. C. and Susan Eastman Newcombe Fellowship in Plant Physiology.

[3] Paper from the Department of Botany, University of Michigan, No. 1046.

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growth period in order to prevent contamination by bacteria and fungi. Bacterial counts taken by plating on nutrient agar were extremely low and there was no appreciable increase or none at all during the incubation period probably due to the high osmotic concentrations. After the incubation the pH was adjusted to 3.0 with HCl and the solution extracted overnight with 50 ml of ether in the cold. After 2 washings with fresh ether the combined ether fractions were evaporated to dryness. The residue was analyzed for growth substance by the standard Avena curvature test (20). All tests involving Avena assay are subject to 10 % error due to biological variability.

The hormones in these ether extracts were purified using the $NaHCO_3$ method and analyzed by paper chromatography as described elsewhere (5). All procedures were conducted quantitatively. Isopropanol, ammonia and water in a ratio of 10:1:1 were used as components of the solvent mixture. R_f values were based on Avena curvatures produced by extracts of eluted sections of the chromatogram. These R_f values were then compared to those of synthetic auxins developed with 1 % *p*-dimethylaminobenzaldehyde in 1 *N* HCl.

Experimental Results and Discussion

Tryptophan Changes: The results of table I are typical of three independent experiments and each figure is the average of 3 to 5 replications with standard deviations indicated for replications. The total tryptophan of the styles increases after pollination. This increase might well be due to the large mass of pollen tubes growing through the style and contribution additional cytoplasmic protein. This increase in protein is shown by the figures in the last column of table I. The total tryptophan of the ovary increases in amount after pollination when the values are expressed per ovary. This organ is rapidly growing primarily by cell enlargement and thus increases in tryptophan on a dry weight basis are nullified by increases in cell wall and cytoplasmic substances. Therefore results are expressed per individual part.

However, the free tryptophan data are more pertinent to the problem of "limiting precursor" since this is part of the amino acid pool that is active in metabolism. Free tryptophan of the style decreases in amount immediately after pollination. This decrease corresponds precisely with the increased rate of IAA synthesis in the same tissue (5). This suggests tryptophan as an active participant in the synthesis of hormones in the style—a synthesis which is apparently induced by the pollen tubes growing through the style. After this initial period, synthesis of tryptophan catches up with utilization and continues to increase. Further research with respect to the biosynthesis of tryptophan would be worthwhile, since little is known about its synthesis in higher plants. There is the possibility that it arises from such substances as indole and serine (10), anthranilic acid (16), or skikimic acid (2).

On a per ovary basis (table I) there is an increase in the level of free tryptophan about 24 and 41 hours after pollination. Shortly after this time the pollen tubes enter the ovary and increased hormone synthesis is observed (5). These data affirm the concepts of Nitsch (13, 14) that tryptophan is associated with the hormone changes taking place during the setting of fruit.

In order to establish whether or not resting pollen contributed free tryptophan to the stigma or style, or if this substance increased in amount in pollen tubes grown in vitro, investigations of the tryptophan content of growing pollen tubes were conducted by germinating 50 mg of pollen in a nutrient medium on a shaking apparatus. The material was then removed from the liquid by centrifugation and boiled with 6 ml of ethanol until the ethanol had evaporated. The residue was then extracted with hot water and an-

Table I

Changes in Concentration of Free and Total Tryptophan in the Styles and Ovaries of Dixie Bright 102 Tobacco Subsequent to Pollination

Material	Hours after pollination	Total tryptophan	Free tryptophan	Protein tryptophan *
		μgm	μgm	μgm
Styles	0	2.58 ± 0.01	1.74 ± 0.15	0.84
Styles	12	2.72 ± 0.06	1.26 ± 0.04	1.46
Styles	24	3.05 ± 0.08	1.28 ± 0.05	1.77
Styles	41	3.47 ± 0.07	2.38 ± 0.07	1.09
Ovaries	0	11.70 ± 0.16	2.89 ± 0.01	8.81
Ovaries	12	12.00 ± 0.33	3.32 ± 0.07	8.68
Ovaries	24	14.04 ± 0.36	4.64 ± 0.10	9.40
Ovaries	41	16.40 ± 0.43	6.63 ± 0.08	9.77
Ovaries	65	19.30 ± 0.24	5.62 ± 0.03	13.68
Ovaries	85	27.10 ± 0.51	6.04 ± 0.12	21.06

Lyophilization was used for preparation of the samples. Results are expressed per style or ovary, with 20 parts being used for each analysis.

* Protein tryptophan obtained by subtracting figures for free tryptophan from those of total tryptophan.

Table II

Concentration of Free Tryptophan in Pollen and Pollen Tubes of Dixie Bright 102 Tobacco in 5 % Sucrose and a Mineral Medium, Germinated 10 Hrs on a Rotary Shaker in the Darkroom at 26 to 28° C

Material	Hours on shaker	L-Tryptophan/initial wt, μGM/MG
	Experiment 167	
Resting pollen	0	0.100
Resting pollen	0	0.093
Pollen tubes	5	0.095
Pollen tubes	13	0.096
	Experiment 162	
Resting pollen	0	0.120
Resting pollen	0	0.129
Pollen tubes	10	0.102
Pollen tubes	10	0.123

alyzed according to Nitsch and Wetmore (15). The data of table II indicate that the free tryptophan pool is not altered during pollen tube growth and that the amount of free tryptophan present is extremely small. This amount is probably insignificant when the amount of pollen that is required for pollination and fruit-set is considered. The data of table I indicated that pollen tubes increase the amount of protein-tryptophan in the style. This, however, is not immediately available for synthetic reactions of the nature of hormone production. The building blocks or amino acids required for such protein synthesis probably come from the style.

HORMONES IN POLLEN AND POLLEN TUBES: By growing pollen tubes in vitro Muir (7) obtained small increases in hormone as a result of pollen tube growth on a sucrose-agar medium. Nitsch (12) obtained larger increases by supplying tryptophan to the culture medium. The ability of pollen tubes of tobacco to convert exogenously supplied tryptophan to hormone was tested by growing 30 mg of pollen in 20 ml of sucrose-mineral medium with some lots containing 2.5 mg L-tryptophan and some without. Figure 1 illustrates that there is no change in the auxin level when tryptophan is omitted, but when tryptophan is added there is a marked synthesis of hormone. These same responses were observed in five repetitions. The three-hour lag period corresponds to the time of emergence of the pollen tubes. Similar results were obtained with a sterile medium. Attempts to sterilize pollen were unsuccessful and resulted in loss of viability. However, bacterial counts in two such experiments revealed that this medium did not support

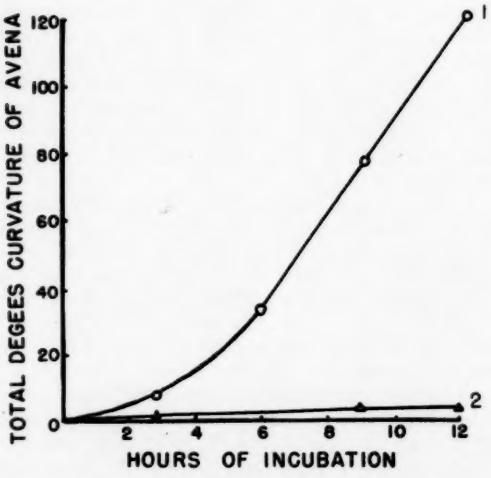


FIG. 1. The synthesis of growth hormones from L-tryptophan by pollen tubes of Maryland Mammoth tobacco as they grow in a liquid medium. Thirty mg of pollen grown in 20 ml of solution in the dark at 28° ± 2° C. Curve 1. With 2.5 mg of tryptophan in culture. Curve 2. Without tryptophan.

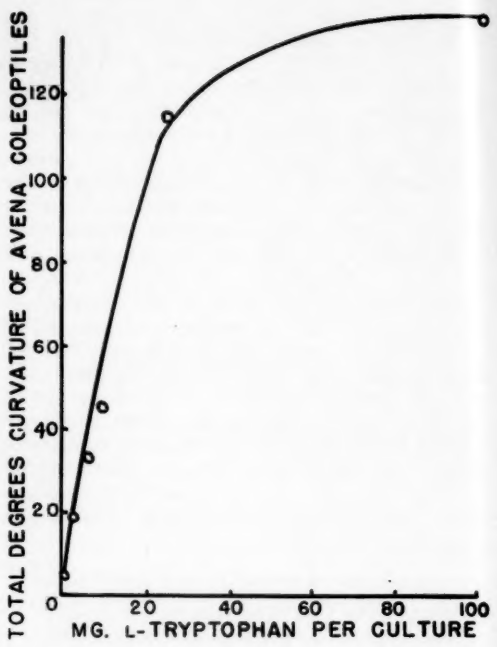


FIG. 2. The effect of concentration of L-tryptophan on the synthesis of growth hormone by pollen tubes of Maryland Mammoth tobacco. Thirty mg of pollen grown in 20 ml of solution in the dark at 28° ± 2° C for 8 hrs.

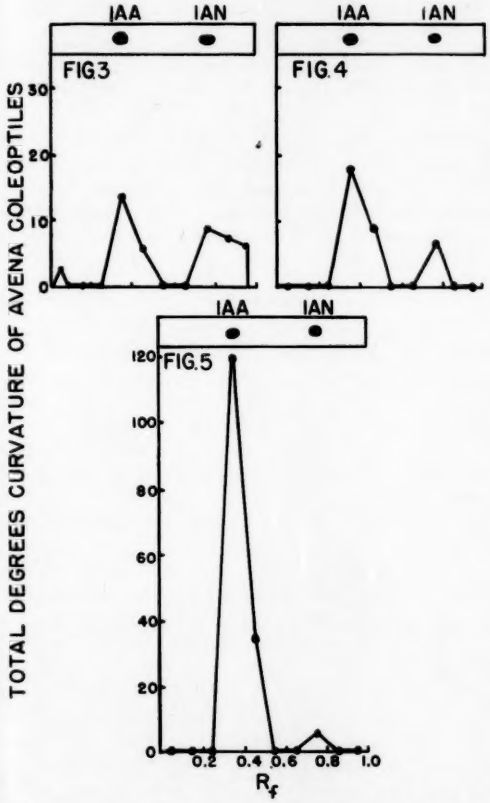
the growth of bacteria and in fact was toxic to them. Tryptophan incubated without pollen on the rotary shaker and in a non-sterile medium for a similar length of time was not converted to auxin.

It is concluded that pollen tubes possess an enzyme system capable of catalyzing the conversion of this amino acid to a substance of growth-promoting activity. This observation fits with the above data on the disappearance of tryptophan in the style shortly after pollination. An active conversion is taking place at the time of pollen tube entry into the style with tryptophan being supplied by the style.

In order to test the effect of concentration of substrate on this system 20 mg of pollen were added to separate culture media with varying concentrations of L-tryptophan and placed on the rotary shaker in the dark for 8 hours at 27 to 28° C. The hormone produced was extracted with peroxide-free ether at pH 3.0 and the extract analyzed by the Avena curvature test. The results of two such experiments as are typically illustrated in figure 2 yielded the usual enzyme-saturation curve.

SEPARATION AND IDENTIFICATION OF HORMONES IN POLLEN AND POLLEN TUBES: Partially purified ether extracts of resting pollen were subjected to paper-chromatographic analysis using Avena curvatures as a criterion of the presence of the hormones separated

on the paper. The methods used are described in an earlier paper (5). All report experiments have been repeated at least twice and again with another solvent (*n*-butanol–ethanol–water, 4:1:1). Figure 3 indicates that one of the growth-promoting substances in resting pollen coincides precisely with the R_f of pure indoleacetic acid. The other area of growth activity on the chromatogram corresponds to the R_f of indoleacetonitrile (IAN). Repeated observation demonstrated IAA to be present in all extracts, but that the absolute identification of IAN needs further verification. This is another confirmation as to the presence of IAA in pollen. Müller (9) found IAA to be the growth hormone in the pollen of orchids. The above data also bring forth realization that the hormones in the pollen and styles (5) are not different as was considered earlier (8), but are the same, namely indoleacetic acid.



FIGS. 3 to 5. Diagrams showing distribution of growth hormones on a paper chromatogram of extracts of pollen of Maryland Mammoth Tobacco. Above the graphs are shown the R_f values of IAA and IAN chromatographed at the same time.

FIG. 3. Resting pollen, 185 mg.

FIG. 4. Pollen (185 mg) germinated in 50 ml of solution 10 hrs on shaker at 28° ± 1° C.

FIG. 5. Pollen (185 mg) germinated 10 hrs on shaker at 28° ± 1° C with 6.25 mg L-tryptophan in 50 ml of solution.

After germination of tobacco pollen a quantitative extraction and chromatographic analysis revealed a peak of growth-activity again corresponding to IAA (fig 4). The height of the IAA-peak had increased by 25 % and the hormone with the higher R_f had decreased in amount.

An additional experiment was conducted to confirm the previously made assumption that IAA is the growth-promoting substance observed in extracts of pollen tubes grown in vitro in the presence of tryptophan. Again chromatographic analysis of pollen tubes grown in 2.5 mg of L-tryptophan per 20 ml of medium revealed that a growth-substance with an R_f that is identical with that of IAA is present in large amounts (fig 5). The original weight of the pollen used was similar to those of figures 3 and 4. Thus, the product of tryptophan oxidation in pollen tube cultures is indoleacetic acid.

TRYPTOPHAN-IAA ENZYME SYSTEM IN POLLEN AND POLLEN TUBES: Heretofore only the resting pollen of flowers has been studied for tryptophan-IAA converting enzymes (13). Therefore, an attempt was made to grow pollen tubes and study enzyme preparations of these structures.

To do this, large quantities of tobacco pollen, usually 200 mg were added to 50 ml of 10 % sucrose and a mineral culture medium. After incubation on a rotary shaker for 8 to 10 hours the cultures were centrifuged and the viscous mass of pollen tubes were homogenized in 1/15 M Na_2HPO_4-KH_2PO_4 buffer at pH 7.0. The pollen tubes were satisfactorily broken in a small homogenizer consisting of a motor-driven

TABLE III

TRYPTOPHAN-IAA ENZYME ACTIVITY IN HOMOGENATES OF POLLEN AND POLLEN TUBES *

SOURCE OF ENZYME	L-TRYPTOPHAN, MG	TOTAL DEGREES CURVATURE OF AVENA COLEOPTILES			
		EXPT 149 **	EXPT 160 †	EXPT 161 ††	EXPT 163 ††
Resting pollen	0	0	...	4.8	0
Resting pollen	2.5	10.2	...	14.4	14.6
Pollen tubes	0	0	6.5	0	0
Pollen tubes	2.5	40.0	47.4	45.9	66.0
Pollen tubes grown in tryptophan	0	...	5.2	0	...
Pollen tubes grown in tryptophan	2.5	...	61.5	58.8	...

* Incubated in a mixture containing 10 ml of 1/15 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, with and without tryptophan. Avena assay after 5 hrs incubation in the dark at 28° C.

** Experiment 149, fresh pollen and pollen tubes of Maryland Mammoth.

† Experiment 160, lyophilized pollen tubes of Dixie Bright 102.

†† Experiments 161 and 163, fresh pollen and pollen tubes of Dixie Bright 102.

pestle made of plastic which rotates in a glass tube. The system was immersed in an ice bath during this procedure. Aliquots were incubated with and without L-tryptophan (2.5 mg/10 ml) in the dark at 28° C. After 5 hours the preparations were adjusted to pH 3.0 and extracted with 2 to 3 washings of peroxide-free ether and analyzed for hormone by the Avena curvature test.

In table III it is seen that resting pollen contains a small amount of enzyme capable of converting tryptophan to IAA, but that the pollen tubes contain much more calculated on the basis of the original weight of the pollen. Incubation mixtures containing tryptophan but no enzymes give no curvature when extracts are applied to Avena coleptiles. Thus, after pollen germination additional enzymes are produced which are capable of IAA synthesis from tryptophan.

Coincident with the preceding experiments, the enzyme system of pollen tubes grown in a medium supplemented with 2.5 mg of L-tryptophan per 20 ml of solution, were analyzed. The pollen tubes were washed twice in fresh sucrose and mineral solution to remove residual IAA and tryptophan before being homogenized. After incubation the enzyme activity of such preparations was found to exceed those of pollen tubes grown on sucrose-mineral medium alone (table III). These data are suggestive of an enzymatic induction by a substrate, in this case, tryptophan. However, even though it is tempting to suggest that this is an induced enzyme, the caution should be taken that this may represent a difference in growth rate. In any event such substances would be supplied by the style to the developing pollen tubes with the effect of producing a larger quantity of enzymes bringing about IAA synthesis. This effect would increase and magnify as the pollen tubes grew through the style and into the ovary. Certainly further investigation of this possibility of induced enzyme synthesis should be investigated.

TRYPTOPHAN-IAA ENZYMES IN STYLES AND OVARIES: This same enzyme system was studied in the styles and ovaries of unpollinated flowers and flowers which had been pollinated at various time intervals and collected on the same day. The material was quick frozen in dry ice–methyl cellusolve mixture and lyophilized until dry. The dried material was ground to pass a 60-mesh screen in the Wiley Mill. Weighed aliquots were added to 10 ml of 1/15 M Na_2HPO_4-KH_2PO_4 buffer at pH 7.0. To some of the tubes, 2.5 mg of L-tryptophan was added. After incubation at 28° C in the dark, the contents were adjusted to pH 3.0 and extracted with 3 washings of fresh ether and analyzed for hormone by the Avena curvature assay.

Styles and ovaries from unpollinated and pollinated flowers contain an enzyme system capable of IAA synthesis from L-tryptophan (table IV). The activity of this enzyme increases after pollination in the style both on the basis of unit dry weight and per flower. This demonstrates that pollen tubes increase the synthesizing activity when grown in vivo as well as in vitro. The activity in the ovaries remains unchanged until about 46 hours after pollination, when there is a marked increase which continues for several days. The increase in activity could easily account for the production of hormones active in the enlargement of the ovary.

TABLE IV

INCREASE IN THE TRYPTOPHAN CONVERTING ENZYME IN STYLES AND OVARIES FOLLOWING POLLINATION OF FLOWERS OF THE DIXIE BRIGHT 102 TOBACCO. THE INCUBATION TOOK PLACE IN THE DARK AT 28° C FOR A PERIOD OF 5 HRS

MATERIAL	HOURS AFTER POLLINATION	TOTAL DEGREES CURVATURE PER AVENA COLEOPTILE			
		NO TRYPTOPHAN		WITH TRYPTOPHAN	
		PER 100 MG DRY WT	PER STYLE OR OVARY	PER 100 MG DRY WT	PER STYLE OR OVARY
Styles	0	0	0	10.8	0.310
Styles	24	0	0	27.9	0.966
Styles	46	2.1	0.72	34.8	1.200
Ovaries	0	15.4	1.85	20.4	2.46
Ovaries	24	0	0	22.4	2.95
Ovaries	46	0	0	30.0	4.11
Ovaries	72	0	0	32.4	6.75
Ovaries	96	0	0	27.3	9.90
Ovaries	118	0	0	36.8	18.78

SUMMARY

The levels of free tryptophan in the styles and ovaries of tobacco were followed. Immediately after pollination the level decreases in the styles and then begins to rise beyond the amount found in styles of unpollinated flowers. The free tryptophan of the ovary increases 24 to 41 hours after pollination. The pollen tubes of tobacco can be grown in a liquid, sucrose-mineral solution on a rotary shaker. Under such conditions they can convert supplemented tryptophan to a growth hormone. This is dependent upon time and concentration of substrate. The hormone of resting and germinating pollen was identified as indoleacetic acid by paper chromatography. Indoleacetic acid was also identified as the hormone produced by pollen tubes in the presence of tryptophan. The enzymatic activity as measured by the ability to convert tryptophan to indoleacetic acid increases 3-fold in homogenates of pollen tubes as a result of 10 hours germination and 4-fold when pretreated with tryptophan. The same enzyme system increases in activity in the styles immediately after pollination and somewhat later in the ovaries. It is concluded that the pollen tubes of tobacco supply to the style and ovaries additional amounts of enzymes which oxidize tryptophan to IAA and hence initiate fruit development.

The author wishes to acknowledge the help and criticisms of Dr. F. G. Gustafson during this investigation.

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INTERMEDIATES OF THE PENTOSE PHOSPHATE PATHWAY AS RESPIRATORY SUBSTRATES[1,2]

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In the past few years a considerable amount of evidence has accumulated in favor of the participation of reactions of the pentose phosphate pathway in the carbohydrate metabolism of plants. This evidence, which has been summarized recently by Axelrod and Beevers (1), has come both from work on extracted enzyme systems and from a variety of experiments on intact tissues. Among the latter may be mentioned those which have demonstrated that the interconversion of labeled sugars, the incorporation of fragments of labeled sugars into other compounds, and the respiratory utilization of glucose labeled in specific positions occur in many cases in a fashion which is predictable from the operation of the pentose phosphate cycle.

To facilitate presentation of the results and discussion an outline of the major reactions of the pentose phosphate cycle is shown in figure 1. A detailed consideration of the individual reactions as they occur in plants is given elsewhere (1). The essential features of the cycle, represented in figure 1 as beginning with 6 molecules of glucose-6-phosphate are: (a) oxidative and decarboxylating steps at the aldehyde end in which glucose-6-phosphate becomes converted first to 6-phosphogluconate and thence to pentose-5-phosphate; and (b) a sequence of splitting and transfer reactions brought about by transketolase and transaldolase which results in the conversion of the pentose

[1] Received March 9, 1956.

[2] This paper is based on work performed under contract No. AT(11-1)-330 with the Atomic Energy Commission.

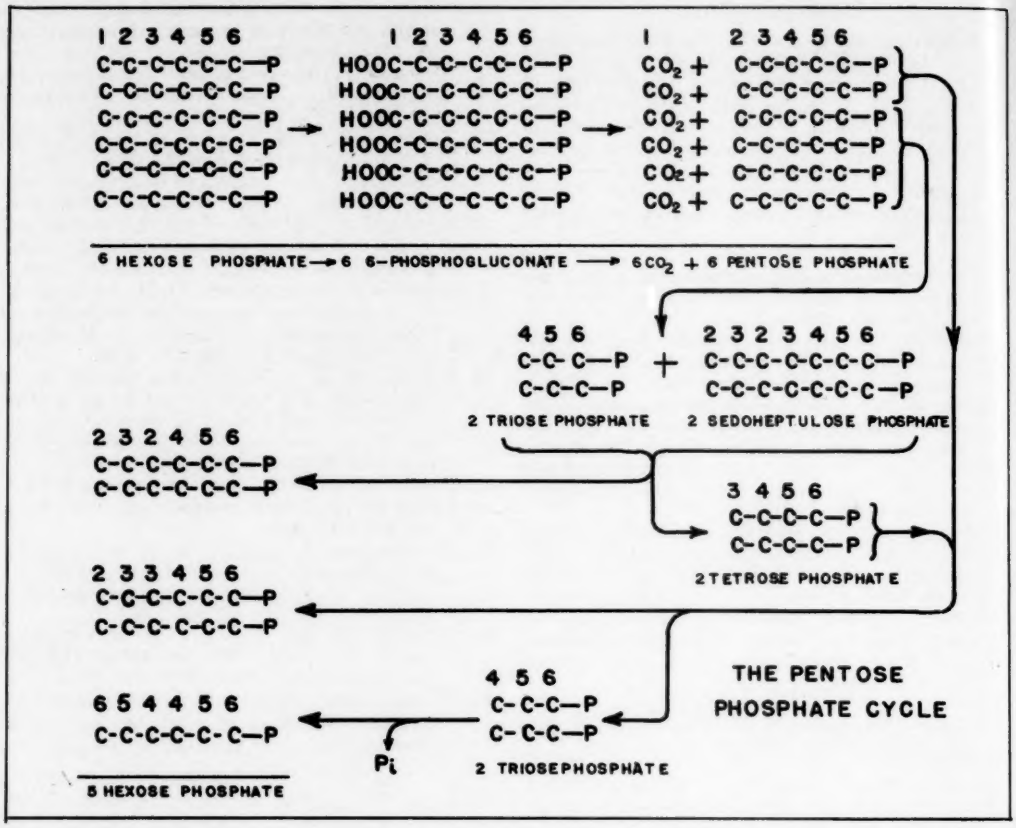

FIG. 1. Diagrammatic representation of the major reactions of the pentose phosphate cycle. The numbers refer to the positions occupied by the carbon atoms in the original hexose phosphate molecules.

phosphates into hexose phosphates, with sedoheptulose phosphate as an important intermediate.

The successful demonstration of these reactions in cell free systems from plants and particularly the work of Gibbs and Horecker (7) using specifically labeled pentose phosphate as a substrate for pea root preparations have given good grounds for supposing that the path taken by the individual C-atoms is essentially that shown in figure 1.

The fact that several tissues have given evidence of metabolizing supplied glucose by reactions in which C-1 makes an early contribution to the CO_2 (3, 6) and the successful demonstration of the oxidation of 6-phosphogluconate and pentose-5-phosphate by pea extracts (5) encouraged the hope that further evidence of the operation of the pentose phosphate pathway in vivo might be obtained by investigating the utilization of intermediates in the reaction sequence.

In the present report then, experiments are described in which intermediates of the cycle (or more correctly the parent compounds corresponding to some of the phosphate esters shown in figure 1) were provided to respiring plant tissues, and their utilization followed by techniques similar to those used in the glucose experiments (2, 3). C^{14}-labeled gluconic acid, the pentoses D-ribose and D-xylose, and sedoheptulose were used in order to find (a) whether they were metabolized in air and under nitrogen and (b) whether anything could be deduced as to the mechanism of their utilization.

MATERIALS AND METHODS

PLANT MATERIAL: Young corn coleoptiles from 2- to 3-day-old seedlings of variety Wf9/38-11 were used in many of the experiments. In addition, tissues of pea seedlings (Alaska) grown on sand and for comparison a variety of other plant materials, mentioned in the text, were employed.

C^{14}-LABELED COMPOUNDS: D-Xylose and D-ribose, labeled specifically in the C-1 position were supplied by the National Bureau of Standards (Dr. H. S. Isbell). The sodium salts of gluconate-1-C^{14}, gluconate-2-C^{14} and gluconate-6-C^{14} were prepared from the corresponding labeled glucose (N.B.S.) by the method of Moore and Link (10). Sedoheptulose, uniformly labeled with C^{14} (sedoheptulose-U-C^{14}) was

generously supplied by Dr. N. E. Tolbert, Oak Ridge National Laboratory. It had been separated from Sedum which had received $C^{14}O_2$ (15).

Usually 20 to 50 micromoles of labeled substrate was shaken at 25° with about 1 gm of thinly sliced tissue suspended in 3 to 4 ml of a solution containing 0.5 ml 0.1 M phosphate at pH 5.0. Large (100-ml) Warburg flasks were employed, and the CO_2 was collected in CO_2-free KOH and converted to $BaCO_3$ at the end of the experimental run. It was then washed, plated and dried and its radioactivity determined in a windowless gas flow counter. The figures in the tables are in counts per minute (cpm) corrected for self absorption and background.

In the anaerobic experiments the substrates were added from the side arms of the flasks after flushing with purified nitrogen for 15 min and a further 15 min equilibration period. When alcohol was collected it was converted to acetate as described earlier (2). The acetate was then divided into two equal samples, one of which was used for a total oxidation (13) and the other for the determination of radioactivity in the COOH group by the Schmidt reaction (12). The total activity in the CH_3 group was then calculated by difference. For experiments in which alcohol produced under aerobic conditions was required, arsenite was added (2). In all cases, the use of large amounts of tissue resulted in yields of CO_2 and alcohol which were large enough to render the addition of carriers unnecessary. The high endogenous rates of respiration were not noticeably increased by the addition of any of the substrates used in the experiments.

Results

Pentose Experiments, *Aerobic:* Table I summarizes the results of a series of experiments in which D-xylose or D-ribose was supplied in air to a variety of tissues and the respiratory CO_2 collected. In most cases the experimental period was 4 hrs. It will be seen that all of the tissues are capable of converting C-1 of both of the supplied pentoses to CO_2 although some of the rates were quite low. In general, the more actively respiring tissues were more efficient in using the pentoses. Figure 2 shows the effect of increasing the amount of corn coleoptile tissue at fixed concentrations of ribose and zylose. It will be seen that the respiration of each pentose proceeds in an orderly fashion, with ribose the slightly more effective substrate. The response of a given quantity of tissue to increasing substrate concentration is shown in figures 3 and 4. Again there is a clear parallel in the effects of the two substrates. Ribose is again somewhat more rapidly utilized but half maximum rates of $C^{14}O_2$ release are obtained at roughly the same substrate level (15 micromoles in 2 ml).

Table I

Aerobic Experiments with D-Xylose-1-C^{14} and D-Ribose-1-C^{14}

Material	Pentose supplied			% Activity recovered
	Ribose	Xylose	Activity	
	micromoles		*cpm* $\times 10^3$	$CO_2/hr \times gm$ *fresh wt*
7-day Pea				
Oldest internode	50	..	40.0	0.56
" "	..	50	21.5	0.52
Primary root tips	20	..	11.0	3.23
" " "	..	20	8.6	2.61
Secondary root tips	2	..	0.86	7.80
" " "	5	..	2.15	6.55
" " "	10	..	4.3	4.35
" " "	20	..	8.6	3.75
" " "	50	..	21.5	2.24
4-day Corn				
Primary root tips	20	..	16.0	1.28
" " "	..	20	8.6	1.61
Root, second 1.5 cm	20	..	16.0	1.02
" " " "	..	20	8.6	1.22
5-day Corn				
Coleoptiles	20	..	11.0	2.47
"	..	20	8.6	3.93

Table II

Anaerobic Dissimilation of D-Xylose-1-C^{14} (Experiments in Which the Distribution of Tracer in the CO_2 and Alcohol Accumulating under Nitrogen Was Determined)

Material		Duration of expt	Xylose added		Activity recovered			Ratio CH_3/CO_2
Type	Amt		Amt	Activity	CO_2	CH_3 of alcohol	CH_2OH of alcohol	
	gm	*hrs*	*micromoles*	*cpm* $\times 10^3$	*cpm*	*cpm*	*cpm*	
Corn								
Root tips	1.3	5	30	16.05	292	565	55	1.93
Coleoptiles	0.85	6	30	16.05	1370	2685	65	1.95
"	1.4	5.5	30	16.05	1711	4292	43	2.5
Pea								
Root, 5 da	2.0	5.5	50	26.75	201	405	10	2.02
" 3 da	2.5	6	50	26.75	33	800	3	2.42
Plumule, 3 da	2.5	6	50	26.75	61	111	9	1.83
Carrot								
Root discs	3.0	8	50	26.75	130	290	10	2.22

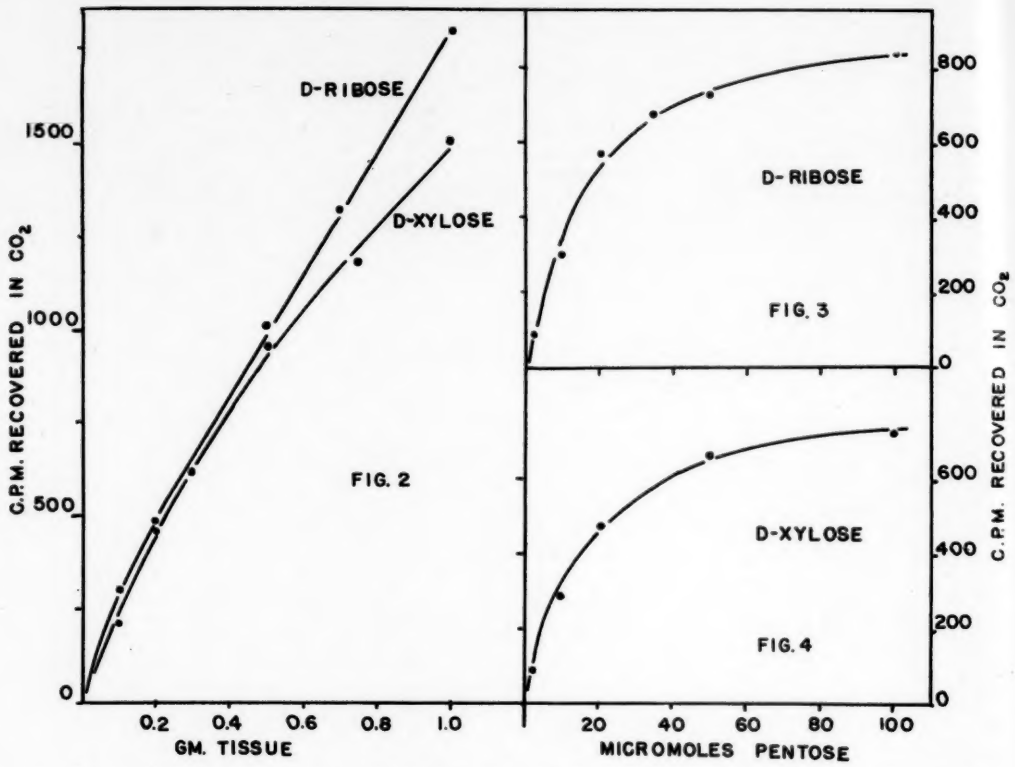

FIG. 2. Respiration of labeled pentoses by corn coleoptile tissue. The effect of varying the amount of tissue on the yield of $C^{14}O_2$ from 45 micromoles of D-ribose-C^{14} (25,000 cpm supplied) and D-xylose-1-C^{14} (32,200 cpm supplied). Experimental period 280 min.

FIGS. 3 and 4. The effect of increasing the amount of D-ribose-1-C^{14} (555 cpm/micromole) and D-xylose-1-C^{14} (715 cpm/micromole) on the radioactivity recovered in the respiratory CO_2 from 300 mg tissue. Experimental period 280 min.

Anaerobic: Radioactive CO_2 was also released when certain of the tissues were provided with labeled pentoses under nitrogen (column 6 in table II and table III). By far the most active tissue in this regard was the corn coleoptile; this is also the tissue which has the highest and most prolonged rate of endogenous CO_2 release. An important feature of the results was that in every tissue, the alcohol which was produced, as well as the CO_2, was labeled. When this is taken into account it is seen that in the case of the corn coleoptile up to 40 % of the ribose and about 30 % of the xylose had appeared in these two fractions at the end of a 5- to 6-hr experimental run. Of greater significance is the fact that the pattern of labeling in the alcohol recovered is the same in each case (tables II and III). The radioactivity was very largely confined to the CH_3 group and in only one experiment had the CH_2OH group more than 10 % of the total counts in the alcohol.

Moreover, as a comparison of columns 6 and 7 in these tables shows, there appears to be a fairly constant relationship between the total activity recovered in the CH_3 of the alcohol and that in the CO_2. The figures in the final columns show that this value is interestingly close to 2 in the majority of the experiments.

Aerobic Experiments with Arsenite: Table IV shows the results of experiments in which the aerobic accumulation of alcohol was induced by the addition of 0.005 M arsenite. Since aerobic respiration is only partially inhibited under these conditions (2) the CO_2 has a dual origin, and only part of it corresponds to the alcohol which accumulates. It is clear, however, that the alcohol is labeled, and the activity is again largely confined to the CH_3 group, although a significantly higher amount is found in the CH_2OH group than was so in the aerobic experiments.

GLUCONATE EXPERIMENTS: Table V shows that gluconate-1-C^{14} was utilized effectively by both coleoptile and root tissues, but that in nitrogen, the release of $C^{14}O_2$ was strikingly reduced. Having established that coleoptile tissue was capable of metabolizing gluconate, a series of experiments was carried out in which equivalent amounts of gluconate-1-C^{14},

TABLE III

ANAEROBIC DISSIMILATION OF D-RIBOSE-1-C^{14}
(EXPERIMENTS IN WHICH THE DISTRIBUTION OF TRACER IN THE CO_2 AND ALCOHOL ACCUMULATING UNDER NITROGEN WAS DETERMINED)

MATERIAL		DURATION OF EXPT	RIBOSE ADDED		ACTIVITY RECOVERED			RATIO CH_3/CO_2
TYPE	AMT		AMT	ACTIVITY	CO_2	CH_3 OF ALCOHOL	CH_2OH OF ALCOHOL	
	gm	*hrs*	*micromoles*	*cpm* $\times 10^3$	*cpm*	*cpm*	*cpm*	
Corn								
Root tips	1.3	5	30	10.8	180	342	48	1.90
Coleoptiles	0.85	6	30	10.8	1075	3442	66	3.04
"	1.4	5.5	30	10.8	1437	3325	60	2.32
Pea								
Root, 5 da	2.0	5	50	18	171	191	17	1.12
" 3 da	2.5	6	50	18	58	93	6	1.60
Plumule	2.5	6	50	18	156	223	8	1.43
Carrot								
Root discs	3.0	8	50	18	162	267	18	1.65

gluconate-2-C^{14} and gluconate-6-C^{14} were the substrates. The results of such an aerobic experiment are shown in figure 5. It will be seen that from C-1 labeled gluconate, the $C^{14}O_2$ release starts out rapidly and is maintained at this high level, while that from the C-2 and C-6 labeled material occurs at much slower and somewhat increasing rates. This indicates of course that C-1 is removed in an early reaction which occurs more rapidly than those which result in the subsequent release of C-2 and C-6.

Anaerobic experiments in which alcohol was collected were carried out with the same substrates even though it was clear from table V that under these conditions the metabolism of gluconate was limited. The results are shown in table VI and the following points may be noted.

(a) The yield of $C^{14}O_2$ is again highest from the gluconate-1-C^{14}; that from gluconate-2-C^{14} was much smaller, but this in turn was noticeably larger than that from gluconate-6-C^{14}, which was extremely small.

(b) The alcohol produced from gluconate-1-C^{14} was essentially unlabeled, in contrast to that from both C-2 and C-6 labeled substrates.

(c) The radioactivity of the alcohol produced in these cases was confined to the CH_3 group.

SEDOHEPTULOSE EXPERIMENTS: Results from experiments on the utilization of sedoheptulose-U-C^{14} are shown in table VII. It is clear from these that, both in air and nitrogen, this substrate is metabolized, and the yields of $C^{14}O_2$ under the two conditions are not greatly different. The alcohol produced anaerobically is also labeled, and as shown, the radioactivity is fairly equally divided between the two carbon atoms. An unsuccessful attempt to increase the utilization of sedoheptulose by the addition of (unlabeled) pentose is also shown in table VII.

DISCUSSION

The demonstration that pentoses, gluconate and sedoheptulose can be used as respiratory substrates by plant tissues can not in itself be considered as evidence of the occurrence of the pentose phosphate pathway, but it is clearly consistent with the operation of such a reaction sequence. The particularly active utilization of the pentose by corn coleoptiles indicates that, by whatever pathway the breakdown is achieved, the capacity of the system may be quite considerable.

Evidence as to the mechanism of pentose utilization is provided by the data from the anaerobic ex-

TABLE IV

AEROBIC DISSIMILATION OF PENTOSES IN THE PRESENCE OF 0.005 M ARSENITE

MATERIAL		DURATION OF EXPT	PENTOSE ADDED			ACTIVITY RECOVERED		
TYPE	AMT		RIBOSE	XYLOSE	ACTIVITY	CO_2	CH_3 OF ALCOHOL	CH_2OH OF ALCOHOL
	gm	*hrs*	*micromoles*		*cpm* $\times 10^3$	*cpm*	*cpm*	*cpm*
Corn								
Coleoptiles	1.2	5	30	..	12.9	....	1057	153
"	0.85	6	30	..	10.8	1400	456	84
"	0.85	6	..	30	16.05	2300	811	99
Root tips, 3 da	1.3	6	30	..	10.8	477	161	145
" " " "	1.3	6	..	30	16.05	623	226	88

TABLE V

RESPIRATION OF GLUCONATE-1-C^{14} BY CORN ROOT TIPS AND COLEOPTILES

GAS PHASE	CPM × 10^3 ADDED	CPM RECOVERED	
		COLEOPTILES	ROOT TIPS
Air	24.8	3520	1930
N_2	24.8	512	250

Each material (0.9 gm) was incubated for 7 hrs with 50 micromoles gluconate-1-C^{14}.

periments with ribose-1-C^{14} and xylose-1-C^{14}. In these, it will be recalled, the CO_2 and the alcohol which were produced were both labeled; the CH_3 group of the alcohol accounted for almost all of the activity in the alcohol and contained roughly twice that found in the CO_2. Clearly no *simple* cleavage of a pentose chain singly labeled in a terminal position would give rise to alcohol and CO_2 both containing radioactivity, and on this evidence alone some more complex breakdown pattern of the pentose must be suspected. It will now be shown that the results are singularly well explained by supposing that the pentose does in fact undergo the rather complex sequence of reactions shown in figure 1.

It will be seen from this figure that the net result of the reactions subsequent to pentose formation is the production of 4 moles of glucose phosphate and 2 moles of triose phosphate (which are shown in fig 1 as giving rise to a further hexose unit). The positions which the original C-atoms of the pentose would be predicted to take in the resulting hexoses are also indicated in the figure. Of the five hexoses produced, only four contain C-1 of the pentose. In two of these hexose units, C-1 of the pentose (the carbon atoms 2 in fig 1) appears twice, i.e., in the C-1 and C-3 position. These are the hexoses which are produced by the transfer of carbons 1, 2, 3, as a unit, from the sedoheptulose to triose phosphate. Since carbons 1–3 of the sedoheptulose originated from the transfer of C-1 and C-2 from pentose to another pentose unit, both carbons 1 and 3 of the sedoheptulose are derived from C-1 of the pentose. The other two hexoses containing C-1 of the pentose are those produced during the transfer of C-1 and C-2 from the pentose, to a tetrose unit from sedoheptulose. This tetrose unit, like the triose mentioned above, is derived from the lower carbons of the original pentose, and does not contain any C-1. Thus, considering the four hexose units together, all of them would have C-1 derived from C-1 of the pentose and in addition half of them would have C-3 derived from C-1 of the pentose. The average distribution of the original C-1 of the pentose in any pyruvate produced during the action of Embden-Meyerhof-Parnas enzymes on this hexose would then be 2/3 in the CH_3 group, and 1/3 in the COOH group, and if the pyruvate were converted to CO_2 and alcohol, the CH_3 of the alcohol would contain 2/3 of any of the C-1 in the products, and the CO_2 would contain the rest.

Thus if pentose which was specifically labeled in the C-1 position followed exclusively the reactions shown in figure 1 and the resulting labeled hexose phosphates were then broken down to pyruvate, and if the conditions were arranged, as they were in our experiments, so that such pyruvate was converted to alcohol and CO_2, the theoretical expectation would be alcohol which was labeled only in the CH_3 group and CO_2 containing half of this activity. The fact that this expectation comes close to being realized in many of the anaerobic pentose experiments reported here is regarded as a highly significant piece of evidence in favor of the operation in vivo of the reaction sequence outlined in figure 1. It will be seen that, to the extent that one is justified in inferring the pattern of labeling in the hexose phosphate from that observed in the alcohol and CO_2, the results agree closely with those described from the pea root preparations of Gibbs and Horecker (7). In their in vitro experiments in which hexose phosphate was produced from C-1 labeled pentose phosphate, roughly 70 % of the activity recovered in the hexose was in the C-1 position and 30 % in C-3.

The work of Neish (11) on utilization of pentose

TABLE VI

ANAEROBIC DISSIMILATION OF GLUCONATE

SUBSTRATE	CPM × 10^3 ADDED	CPM RECOVERED		
		CO_2	CH_3 OF ALCOHOL	CH_2OH OF ALCOHOL
Gluconate-1-C^{14} *	24.8	853	0	22
" 2-C^{14} *	22.9	134	394	29
" 6-C^{14} *	21.6	29	456	24
" 2-C^{14} **	33.5	209	478	42
" 2-C^{14} **	33.5	203	620	33

* Corn coleoptile tissue (1.2 gm) was incubated with 50 micromoles of the named gluconate for 7 hrs.

** Additional experiments in which 1.5 gm tissue were incubated with 70 micromoles gluconate-2-C^{14} for 5.5 hrs.

TABLE VII

RESPIRATION OF SEDOHEPTULOSE-U-C^{14}

GAS PHASE	SUBSTRATE ADDED		CPM RECOVERED		
	TYPE	ACTIVITY	CO_2	CH_3 OF ALCOHOL	CH_2OH OF ALCOHOL
Air	Sedoheptulose-U-C^{14}	10.8	1080	...	...
Air	Sedoheptulose-U-C^{14} + D-xylose (30 micromoles)	10.8	948	...	...
N_2	Sedoheptulose-U-C^{14}	10.8	638	306	262
N_2	Sedoheptulose-U-C^{14} + D-xylose (30 micromoles)	10.8	634	248	274

Corn coleoptile tissue (0.85 gm) was incubated 7 hrs with 64 micromoles sedoheptulose.

in cellulose synthesis also points to the occurrence in vivo of a similar series of reactions and very recently Ginsburg and Hassid (9) have presented some additional results on pentose utilization in wheat which are susceptible of the same interpretation.

In the anaerobic experiments described above, it should be stressed, the O_2-free conditions presumably limited the entry of glucose (including that from the supplied pentose) into the pentose phosphate cycle. In the arsenite experiments, in which the aim was to determine the labeling in the α and β carbons of aerobically produced pyruvate by inducing its diversion to alcohol, it is again clear that the β carbon (CH_3 of alcohol) has by far the greater activity; the appearance of a somewhat greater amount of activity in the CH_2OH group (α carbon of pyruvate) than is observed in the anaerobic experiments might indicate that some recycling and consequent randomization was occurring in air.

In contrast to the pentose results, in which active utilization occurred both in air and N_2, those obtained in the gluconate experiments show that the withdrawal of O_2 has a large depressing effect on the conversion to CO_2. This is in accord with the requirement for O_2 (or an internal system for the sustained regeneration of TPN) if the oxidative decarboxylation reaction shown in figure 1 is to continue unchecked. In trying to ascertain the breakdown pathway of gluconate the results in figure 5 are an important consideration. These show that, both in air and in N_2, C-1 makes a much larger contribution to the respired CO_2 than C-2 or C-6. Such results would be observed if the gluconate were first converted to pentose as in figure 1. The demonstration that carbons 1 and 6 of the gluconate are not equivalent in their contribution to the respired CO_2 establishes that gluconate is not utilized by reduction back to glucose and subsequent breakdown by the E.M.P. sequence.

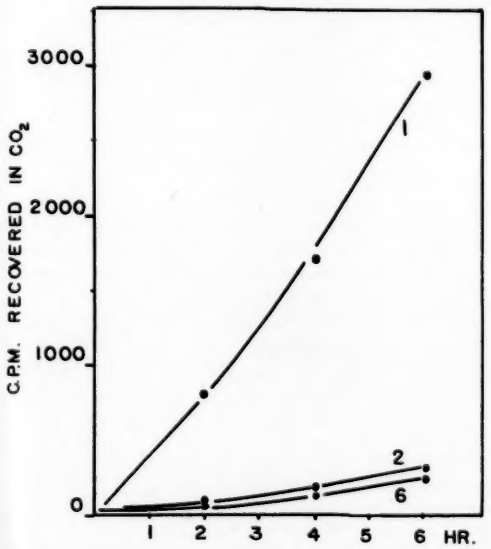

FIG. 5. Respiration of gluconate by corn coleoptile tissue. Fifty micromoles gluconate-1-C^{14}, gluconate-2-C^{14} and gluconate-6-C^{14}, respectively, were provided to samples of 1.3 gm tissue and the respired CO_2 analyzed for radioactivity after 2, 4 and 6 hrs.

Although the anaerobic utilization of gluconate is small, the results of the alcohol degradations in table VI are nevertheless in accord with the regeneration of hexose from gluconate in an oxidative cycle which involves (a) decarboxylation at C-1 and (b) recombination of the pentose carbon by the reactions shown in figure 1 into hexose and subsequent breakdown to pyruvate by the E.M.P. enzymes. As table VI shows, the C-1 of the gluconate made a relatively large contribution to the CO_2 and did not appear in the alcohol. On the other hand, C-6, which would be expected from figure 1 to contribute only to C-1 and C-6 of the hexose and thus the CH_3 group of pyruvate, did not appear in any quantity in the CO_2, and was present, significantly enough, only in the CH_3 group of the alcohol. C-2 of the gluconate would be expected on the basis of figure 1 to become C-1 of the pentose and thus to contribute only to C-1 and C-3 of the hexose in a 2/1 ratio. Table VI shows that in the experiments with gluconate-2-C^{14} the CO_2 (derived from the COOH of pyruvate and equivalent to the C-3 of the hexose) was in fact radioactive but considerably less so than the CH_3 of the alcohol. The CH_2OH group of the alcohol was again unlabeled. The actual ratios of (C^{14} in CH_3 of alcohol/C^{14} in CO_2) observed in the gluconate-2-C^{14} experiments are 2.9, 2.3 and 3.1. Although these values are considered to be reasonably close to 2 in view of the fact that the yield of C^{14} in the products was small, it will be recalled that in some of the pentose experiments values closer to 3 were occasionally observed. This may be taken to indicate that some side reaction not included in figure 1 may occur and result in an additional incorporation of C-1 of pentose into CH_3 of alcohol.

From table V and figure 5 the presumption seems justified that in air the gluconate is metabolized more rapidly but by the same pathway as that taken in N_2.

The fact that pentose utilization occurred readily in our materials in air and N_2 in a way which appeared to include sedoheptulose as an intermediate, made it desirable to investigate the utilization of this compound. Tolbert and Zill (14) had previously observed that in air this material is rapidly utilized and the carbon incorporated into sucrose and a number of other cell components in barley, tobacco and sugar beet leaves but that in N_2 the incorporation was drastically reduced. The results with corn coleoptiles in table VII show clearly that, on the basis of its contribution to respiratory end products (which were not under investigation by Tolbert and Zill), sedoheptulose is metabolized in this tissue both in air and nitrogen. The appearance of radioactivity in the CO_2 produced in air, and in both carbons of the alcohol and

in the CO_2 produced anaerobically are understandable if the sedoheptulose follows the sequence shown in figure 1.

At the outset it had appeared possible that absorption and phosphorylation by the tissues might be so slow as to prevent the active utilization of materials such as pentoses which are not normally thought of as respiratory substrates. In the outcome it was clear that the utilization of all three substrates was at least large enough to allow some firm conclusions to be reached about the metabolism of each.

The conclusions are that, in at least one tissue, the corn coleoptile, the utilization of the pentoses, gluconate and sedoheptulose occurs in a way which, when the possible interference of side reactions is taken into account, is remarkably well accommodated in the coherent picture represented by the sequence of reactions shown in figure 1. Insofar as other tissues have been examined in the critical case of the pentoses, a similar conclusion about the utility of the pentose phosphate pathway seems justified for them also.

It should be made clear that the burden of this report has been to establish the existence of a functional pentose cycle in vivo rather than to reaffirm a respiratory role for the cycle. In fact it will be noticed that the tissue we have found the most convenient for many of the experiments is one of a group of young tissues in which it is clear that the Embden-Meyerhof-Parnas pathway of glycolysis is by far the most important (2, 3, 6).

The utilization of gluconate and the pentoses indicate that, in spite of this, enzymes are present in the tissue which can carry out the reactions of the cycle at least from gluconate onwards. Furthermore Gibbs and his associates (4, 8) have recently shown that enzymes of the cycle, including glucose-6-phosphate dehydrogenase, can be extracted from several very young tissues. In this case, and in others in which enzymes of both pathways (see Axelrod and Beevers (1)) may be shown to be present and active in vivo, the unresolved question concerns the internal influence which presumably determines the immediate fate of the glucose-6-phosphate.

Summary

It has been established that D-xylose and D-ribose can be utilized as respiratory substrates, by a variety of plant tissues. One of the most active of these was the corn coleoptile, and this material was used in further experiments which showed that sedoheptulose and gluconate are also respired. Each of the substrates gives rise to alcohol and CO_2 under nitrogen, although the utilization of gluconate, in contrast to that of the pentoses and sedoheptulose, is markedly reduced under these conditions.

C^{14} appeared both in the alcohol and in the CO_2 produced anaerobically (and aerobically in the presence of arsenite) from ribose-1-C^{14} and xylose-1-C^{14}. In addition, it was observed that the tracer was characteristically distributed in these products; roughly 2/3 appeared in the CH_3 of the alcohol and 1/3 in the CO_2. These findings are interpreted to mean that the pentoses were being metabolized by way of the pentose phosphate cycle.

Experiments with gluconates labeled respectively in the 1-, 2- and 6- positions showed that release of C-1 is an early reaction in its utilization. The pattern of labeling in the alcohol and CO_2 produced anaerobically from the three gluconates is also consistent with its conversion to pentose by loss of C-1 in an oxidative step and subsequent conversion to glucose in the pentose phosphate cycle.

The fact that a plant tissue is capable of respiring gluconate, pentose, and sedoheptulose, which represent intermediates in the breakdown of glucose by the pentose phosphate cycle, is of interest in itself. As evidence for the presence of such a cycle in the tissue a more incisive finding is that the paths taken by individual C-atoms of the labeled substrates, as indicated by the distribution in the products of anaerobic respiration, are essentially those predicted from the present knowledge of the reaction sequence.

The experimental work on which this paper is based was begun while I was a visiting scientist in the Biology Department of Brookhaven National Laboratory in the summer of 1955. I would particularly like to thank Dr. Martin Gibbs of that department for his help and advice during this time as well as for his hospitality.

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LIGHT EFFECTS ON THE GERMINATION OF SEEDS OF HENBIT (LAMIUM AMPLEXICAULE L.) [1,2]

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This paper originated from the observation that seeds of henbit failed to germinate when exposed to light from a north window, but they germinated well in the dark. While the writers have found no literature references to light effects on henbit seed germination, the fact that light affects the germination of certain seeds has been known for many years (3). Certain varieties of lettuce (*Lactuca sativa* L.), especially Grand Rapids, are classic examples of light-sensitive seeds. Flint and McAlister (6, 7) determined that light of wavelengths between 5200 and 7000 Å stimulated the germination of lettuce seed, whereas far-red radiation (7600 Å) sharply inhibited germination of this seed. These observations have been confirmed in more recent work (1, 2) and extended to other species (9).

This study was undertaken to determine the extent to which henbit conforms to the pattern known to exist in other plants. The effects of light intensity, light quality, and the stage of imbibition at which the seeds are irradiated have been investigated.

EXPERIMENTS

Freshly-harvested seeds of henbit (*Lamium amplexicaule* L.) are dormant and will not germinate for several weeks regardless of light conditions. Efforts to break this dormancy with a wide variety of treatments were unsuccessful. A water extract of crushed dormant seeds markedly inhibited the germination of tomato seeds and non-dormant henbit seeds. Apparently the dormant seeds contained a water-soluble inhibitor which decreased during dry storage of the seeds.

The effect of light intensity on germination of non-dormant seeds was established by irradiating seeds on moist filter paper in Petri dishes at various distances from an unfiltered 150-watt incandescent light source. Eleven lots of 50 seeds each were used, one of which was maintained in continuous darkness. Light treatments were continuous for 72 hrs and were followed by an additional germination period of 48 hrs in the dark. Germination counts were made at the end of this light period and again after periods of 24 and 48 hrs in the dark. Weak diffused daylight was used to make the germination counts after the 24-hr dark period.

A major inhibitory effect was obtained with the lowest light intensity used, i.e., 0.8 ft-c (table I). Germination was reduced from 70 to 24 % at this intensity. At 2.8 ft-c germination was completely inhibited. After 24 hrs in the dark, recovery from this light effect was evident in seeds which were treated with less than 2.8 ft-c of light. Forty-eight hours after the light treatment most of the inhibition was gone regardless of the intensity of the irradiation used.

Recovery from light inhibition was noted in another experiment. In this case seeds were presoaked in water for 15 hrs before treatment for four minutes with light from an incandescent source filtered through red and blue Cellophane. This filter combination gave maximum transmittance between 7300 and 8700 Å, with no radiation below 7000 Å. The incident energy of this far-red radiation was approximately 8.1×10^{-3} joules/cm^2 × sec. In this experiment good germination was obtained 120 hrs after the inhibiting light treatment.

Light sensitive seeds generally have shown greater sensitivity to one or more particular parts of the spectrum (1, 3, 5, 6, 7). Henbit seeds show a similar selectivity in their response to light. In these experiments triplicate lots of 100 seeds each were exposed after 16 hrs of imbibition to six minutes irradiation with spectral bands having peak transmissions at 3600, 4300, 5200, 5800, and 6000 Å, respectively. These light treatments were obtained with glass filters (Corning Color Filters, Corning Glass Works, Corning, New York) which were found to transmit spectral bands about 400 Å units wide with peak intensity in the approximate middle of each band. Far-red radiation (7300 to 8700 Å) used in this and subsequent experiments was obtained with light from an incandescent lamp filtered through two layers of red and two layers of blue Cellophane. The incident energies

[1] Received March 13, 1956.
[2] Published with permission of the Director of the Arkansas Agricultural Experiment Station.

TABLE I

EFFECT ON HENBIT SEED GERMINATION OF LIGHT AT VARIOUS INTENSITIES APPLIED CONTINUOUSLY FOR 72 HRS

LIGHT INTENSITY	HRS AFTER LIGHT TREATMENT		
	0	24	48
ft-c	*% Germination*		
0	70	72	82
0.8	24	42	80
1.0	14	26	88
1.2	12	26	80
1.4	12	22	78
2.8	0	0	68
5.2	0	0	70
9.8	0	0	80
13.5	0	0	54
15.5	0	0	52
20.5	0	0	54

at the seed surfaces were similar in all cases, approximately 1.0×10^{-3} joules/cm^2 × sec.

Germination responses of henbit seeds so treated are shown in table II. The major response was to the far-red radiation which reduced germination over 50 %. This identifies henbit with other light-sensitive seeds, such as lettuce and *Lepidium* spp. (1, 9) which are inhibited in germination by far-red radiation. Henbit seed germination was reduced to some extent at 3600 Å (table II). This response to short-wavelength radiation has not been explored further except to verify the fact that no radiation in the far-red could be detected with the filter used.

In these experiments far-red radiation (7300 to 8700 Å) did not prevent the germination of some seeds. Increasing the incident energy as much as twenty times did not reduce germination further. At high energy levels (8.1×10^{-3} joules/cm^2 × sec) increasing the exposure time from 0.5 to 8 minutes resulted in rather small further reductions in germination. Some seeds either were incapable of response to the far-red radiation, or were at a stage of development in which they were insensitive to it.

To find whether the stage of germination was a determining factor in the radiation response, henbit seeds were allowed to imbibe water for periods of 0, 5, 12, and 24 hrs prior to exposure to four minutes of far-red light. Dry seeds were unaffected by this light treatment, but seeds which had imbibed water for 12 hrs gave only 25 % germination, as compared to 70 % for the dark controls. The inhibitory effect of the far-red light was much less with imbibition periods of 5 and 24 hrs. These results agree, in general, with those of Borthwick et al (1) who found that the greatest effect of red and far-red radiation on the germination of lettuce seed was obtained when the seed had imbibed water 4 to 16 hrs prior to irradiation.

TABLE II

GERMINATION OF HENBIT SEEDS, IN LOTS OF 300, AFTER EXPOSURE TO DIFFERENT SPECTRAL BANDS FOR 6 MIN

PEAK WAVELENGTHS IN Å UNITS	% GERMINATION
3600	34
4300	52
5200	49
5800	50
6000	57
Far-red	26
No light	59

Photoreversibility of the germination inhibiting effect of far-red radiation was established for henbit seeds. In a series of treatments similar to those used by Borthwick et al (1) eight lots of 100 seeds each were treated with an alternating sequence of one minute of red (5800 to 7000 Å) and four minutes of far-red (7300 to 8700 Å) radiation after 16 hrs of imbibition in the dark (table III). Red radiation was obtained by filtering light from a white fluorescent source through two layers of red Cellophane. The incident energy of the red radiation was approximately 2.09×10^{-3} joules/cm^2 × sec. The incident energy of the far-red source described earlier was approximately 8.1×10^{-3} joules/cm^2 × sec. Far-red radiation greatly reduced germination, but this effect was completely nullified by a subsequent treatment with red light. Results similar to those presented in table III were obtained in many tests.

TABLE III

GERMINATION OF HENBIT SEEDS, IN LOTS OF 100, AFTER EXPOSURE TO RED AND FAR-RED (FR) RADIATIONS IN SEQUENCE

LIGHT TREATMENT	% GERMINATION
Red	36.9
FR	7.2
Red-FR	3.2
Red-FR-Red	43.6
Red-FR-Red-FR	5.5
Red-FR-Red-FR-Red	48.8
Red-FR-Red-FR-Red-FR	3.9
Red-FR-Red-FR-Red-FR-Red	45.2
No light	42.6

DISCUSSION

Seeds of henbit and certain other plants (1, 3) are light-inhibited in their germination processes. On the other hand some plants, such as Grand Rapids lettuce, require light for germination. The mechanism of response for the two types of light-sensitive seeds appears to be quite different; however, recent investigations (1, 9) have shown that the two types of responses to light depend on the same basic mechanism. Far-red radiation in the 7300 to 7400 Å region inhibits seed germination, while red light, 5800 to 6800 Å, offsets the far-red effect. Henbit seeds have been shown here to be similar to other light-sensitive seeds, since far-red radiation has an inhibitory effect on germination which can be overcome by red light. The

failure of henbit and similar seeds to germinate in ordinary light seems to indicate high sensitivity to the inhibiting effect of the far-red radiation, rather than a unique type of light response.

Henbit seeds are similar to lettuce seeds (1) in the photoreversibility of their response to red and far-red radiation. The pronounced inhibition of germination by far-red radiation can be completely offset by a subsequent exposure to red light. The effect on seed germination of an alternating sequence of the two types of radiation is determined entirely by the final light treatment.

Henbit seeds are sensitive to weak light. The major inhibiting effect of incandescent light was obtained with an intensity of one ft-c (table I), with immediate germination reduced from 70 to 14 %. Higher intensities applied over a period of 72 hrs delayed germination of all seeds treated.

The inhibiting effect of light was found to be temporary, the length of the inhibition period depending to some extent on the intensity of the light used (table I). Seeds irradiated with 13.5 or more ft-c of light for 72 hrs were partially inhibited 48 hrs after the light treatment ended. With lower intensities, recovery was marked in 24 hrs and complete in 48 hrs after the light treatment. Even though low light intensities accomplished the major inhibition, the effects of higher intensities were evident in longer recovery times.

Henbit seeds are sensitive to light only during a limited and early stage in the germination process. Failure of light of short duration to affect germination of all seeds treated after 12 hours of imbibition suggests that not all seeds progress to the critical stage of light sensitivity together. Continuous irradiation of seeds during early stages of germination should provide light during the sensitive period for all germinable seed. This was demonstrated in the first experiment reported here (table I) in which germination of all treated seeds was checked by irradiation begun with the onset of imbibition and continued for 72 hrs. Henbit seeds are similar to lettuce (1) and light requiring tobacco seeds (8) in that dry seeds are unaffected by light and maximum sensitivity to light occurs early in the germination process.

Summary

Freshly-harvested henbit seeds are dormant, apparently because they contain a water-soluble inhibitor which decreases during dry storage of seeds.

The germination of non-dormant henbit seeds is inhibited by light. Light from an incandescent source at an intensity of one ft-c reduced germination from 70 to 14 %. Higher intensities prevented germination of all seeds when applied for 72 hrs to the moistened seeds. The inhibiting effect of light was temporary, the length of the inhibition period depending to some extent on the intensity of light used. Henbit seeds are most sensitive to light after 12 hrs of imbibition of water. Shorter or longer periods of soaking reduce the light sensitivity, and dry seeds are unaffected by light.

Germination of henbit seeds is prevented by far-red radiation (7300 to 8700 Å) and this inhibition is overcome by a subsequent treatment with red light (5800 to 7000 Å). The effect of an alternating sequence of the two types of radiation was determined entirely by the final light treatment.

The authors are indebted to Dr. H. A. Borthwick and Dr. J. L. Bowers for helpful suggestions during the course of this study.

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THE RESIDUAL EFFECT OF AUXIN ON THE CELL WALL[1,2]

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The present paper concerns the nature of the relationship between auxin and the expansion of the plant cell. It will be shown that it is possible to separate in time the action of auxin on the cell from the act of cellular expansion by water uptake.

The separation of auxin action from cell expansion is achieved as follows: auxin is first applied to tissue which is kept from expanding by a suitably high external osmotic concentration. The tissue is then allowed to expand in water under conditions in which the action of auxin is blocked by a suitable inhibitor. An effect of the auxin pretreatment upon the tissue is manifested by an increased expansion of the auxin treated tissue above that of the non-auxin treated control tissue.

Experiments of this nature were first performed by A. N. J. Heyn in 1931 (4) and were continued by Thimann (9). Heyn concluded that the action of auxin results in a loosening of the cell wall. In these experiments, however, expansion was not completely suppressed during the period of auxin action. The possibility remains, therefore, that the effects observed may not have been due to the direct action of auxin but rather indirectly due to some auxin-induced elongation. The present experimental procedure permits a complete separation of auxin action from cell expansion.

METHODS AND MATERIALS

Avena seedlings were grown in the standard manner of McRae and Bonner (8). When the seedlings were 90 to 92 hours old, those with a coleoptile length of 2.75 to 3.25 cm were selected and a 5-mm section cut from each, the apical 2 mm being discarded. The sections were pooled and randomized. Lots of twenty sections were used for each portion of each experiment. Each lot of sections was then subjected to four successive treatments. During an auxin pretreatment or auxin-action period the sections were floated for 45 minutes in 10 ml of 0.3 M mannitol solution buffered with 0.0025 *N* K-maleate and either with or without added indoleacetic acid (IAA), 5 mg/l. This concentration of mannitol is such that at the end of the 45 minutes the length of the sections (measured under a binocular microscope with stage micrometer) is slightly less than the initial length. Plasmolysis does not occur as shown by microscopic examination. In order to assure that the action of auxin was completely blocked before the sections were allowed to expand, the auxin pretreatment was followed by a transition treatment of 30 minutes. This consisted of 10 ml of solution containing 0.3 M mannitol to prevent expansion and, in addition, an inhibitor of auxin action. The sections were then allowed to expand in 10 ml of buffered water containing the inhibitor. After an expansion period of 90 to 120 minutes the sections were removed and measured. The sections were next tested for possible damage by the earlier treatments. They were placed in 10 ml of solution containing IAA, 5 mg/l, and sucrose, 2.25 %, and their growth response determined. If the sections maintained their turgor for 6 to 12 hours, they were assumed to have been not significantly damaged by the earlier treatments.

The experimental results are expressed in terms of ΔL values.

ΔL = Average length of auxin treated sections after expansion – Average length of non-auxin treated sections after expansion.

A ΔL value of 0.06 mm or greater is considered as indicating an effect of auxin which is significant at the 5 % level. This is evident from the data of table I.

TABLE I

UNIFORMITY TRIAL AND DEMONSTRATION OF RESIDUAL EFFECT OF AUXIN ON EXPANSION OF AVENA COLEOPTILE SECTIONS

REPLICATE #	FINAL LENGTHS	
	+ AUXIN	– AUXIN
	mm	*mm*
1.	5.15 ± 0.023	5.03 ± 0.025
2.	5.14 ± 0.021	5.02 ± 0.022
3.	5.14 ± 0.024	5.02 ± 0.015
4.	5.14 ± 0.023	5.02 ± 0.017
5.	5.14 ± 0.021	5.02 ± 0.014
6.	5.14 ± 0.027	5.01 ± 0.017
7.	5.13 ± 0.026	5.01 ± 0.020
8.	5.11 ± 0.025	4.98 ± 0.016
Av.	5.14 ± 0.024	5.01 ± 0.018
ΔL	0.13 mm ± 0.030	

Pretreatment with IAA, 5 mg/l, and 0.3 M mannitol for 45 min. Argon used as inhibitor during transition (30 min) and expansion period (90 min) treatments. Initial length of sections 4.75 mm.

Standard errors were computed for all lots. The average standard error for the auxin treated lots was 0.024 mm while the average standard error for the non-auxin treated lots was 0.018 mm. The standard error of the difference between the means of auxin and non-auxin treated lots was 0.03 mm. A ΔL value of 0.06 mm is, therefore, significant at the 5 % level.

Since, in these experiments, auxin action is separated from cell expansion by a period in which neither takes place, any effect of auxin upon expansion is a residual effect and will be referred to as such.

[1] Received March 20, 1956.

[2] Report of work supported in part by the Herman Frasch Foundation and in part by the National Science Foundation.

Results

Separation of Auxin Action and Cell Expansion: Expansion of coleoptile sections as a result of an earlier auxin pretreatment can be achieved when anaerobic conditions are used as an inhibitor during the expansion period. Auxin-induced growth is inhibited by anaerobic conditions (1). Anaerobic conditions were obtained in the present experiments by the use of argon. After an aerobic auxin pretreatment, the sections were placed in stainless steel wire baskets and immersed in solutions through which argon had been bubbled for at least twenty minutes. This was done for both the transition and expansion period solutions. The anaerobic conditions thus produced should prevent any direct auxin action during the expansion period but should permit any residual auxin effect which is not dependent upon aerobic conditions to express itself as water uptake. The data of table I show that under these conditions the auxin treated sections show a significantly greater expansion than do the non-auxin treated sections. The use of argon as the inhibitor permits expression of a residual auxin effect.

The possibility has been considered that the apparent residual effect of auxin may be due to some artifact of the system and some effort has been made to examine and eliminate such possibilities.

During the transfer of the sections from the transition period solution to the expansion period solution, they are exposed to air for 15 to 30 seconds. During this time it might be possible for the sections to absorb enough oxygen to allow the auxin remaining in the tissues to cause some active growth. This was tested by diluting the 10 ml of transition period solution with one liter of argonated water to constitute the expansion period solution. The results, given in table II, indicate that with this method in which the sections are not exposed even momentarily to air, the residual auxin effect still persists.

The possibility must also be considered that argon does not completely inhibit the action of auxin during the expansion period. If this were the case, the sections should grow when auxin is added during the anaerobic transition and expansion period treatments. The results of such an experiment, shown in figure 1, indicate, however, that the effect of auxin during the anaerobic periods is negligible. Figure 1 also shows

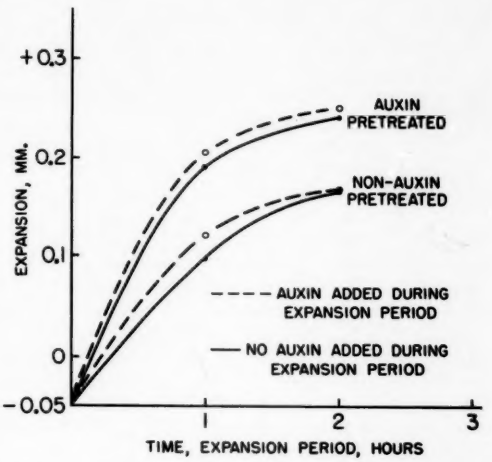

Fig. 1. Time course elongation of Avena coleoptile sections in water and under anaerobic conditions. Sections either pretreated or not pretreated with auxin (IAA, 5 mg/l) and 0.3 M mannitol for 45 min under aerobic conditions, and either treated or not treated with auxin (IAA, 5 mg/l) during the anaerobic expansion period.

that essentially all of the expansion which takes place as a result of the residual auxin effect is consummated within one hour.

When argon is administered during the auxin pretreatment, no residual auxin effect can be detected, affirming the fact that auxin is only effective in the presence of aerobic metabolism (table III).

Evidence for a Cell Wall Stiffening Process: The length of the transition period has been arbitrarily set at 30 minutes in the above experiments. It is necessary that the transition period be sufficiently long to permit suppression of oxidative metabolism. The residual auxin effect appears however to die away if the transition period is excessively prolonged. Thus although a residual auxin effect was found to persist through a transition period of up to one hour, it did not persist through one of 2.5 hours (table IV). Heyn's work (4) has already indicated that when sections are kept in a non-expanding condition for a sufficient length of time, they lose their ability to re-

Table II

Effect of Method of Transference of Sections from Transition to Expansion Period Solutions on the Residual Auxin Effect

Method of transference	ΔL mm	Residual auxin effect
In air	+0.13	+
By dilution	+0.17	+

Argon used as inhibitor of auxin action after auxin pretreatment (IAA, 5 mg/l, in 0.3 M mannitol for 45 min).

Table III

Effect of Anaerobic Conditions During Auxin Pretreatment of Coleoptile Sections

Argon during auxin pretreatment	ΔL mm	Residual auxin effect
No	+0.15	+
Yes	−0.05	−

Argon used as inhibitor of auxin action after pretreatment (IAA, 5 mg/l, in 0.3 M mannitol for 45 min).

TABLE IV

RELATION OF RESIDUAL AUXIN EFFECT TO TOTAL TIME OF SECTIONS IN MANNITOL

PRETREATMENT (NO AUXIN)	AUXIN PRETREATMENT	TRANSITION PERIOD	TOTAL TIME IN MANNITOL	ΔL	RESIDUAL AUXIN EFFECT
	min	*min*	*min*	*mm*	
None	45	30	75	+0.09	+
None	45	150	195	−0.03	−
120 Min	45	30	195	−0.04	−

Argon used as inhibitor of auxin action after auxin pretreatment (IAA, 5 mg/l, 0.3 M mannitol). Expansion period 90 min.

spond to added auxin by expansion. This Heyn interpreted as due to a continuing stiffening of the cell wall.

Heyn's hypothesis is supported by the results of the experiments of table IV. For this experiment sections were treated for two hours in 0.3 M mannitol in the absence of auxin before being subjected to the usual treatments (30-minute transition period). No residual effect of auxin could be detected (table IV). Some sort of stiffening of the tissue apparently takes place during prolonged periods of non-expansion. This stiffening is evidently independent of the presence of auxin in the medium and occurs both during prolonged pretreatment in 0.3 M mannitol and during prolonged transition period treatment.

LENGTH OF AUXIN PRETREATMENT AND SUBSEQUENT EXPANSION: The length of the auxin pretreatment has been arbitrarily set at 45 minutes in the above experiments. Variation in the length of the auxin treatment time, however, produces a proportional variation in the residual auxin effect (ΔL value) as is shown in figure 2. The magnitude of the residual response to auxin is directly proportional to the length of the auxin treatment.

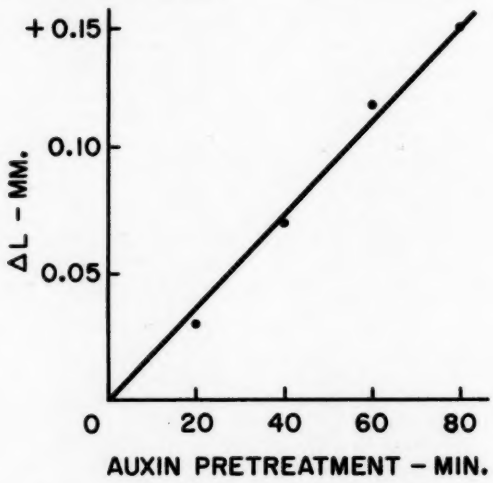

Fig. 2. Relation between residual auxin effect (expansion in water as a result of auxin pretreatment) and length of auxin pretreatment (IAA, 5 mg/l in 0.3 M mannitol). Expansion period 90 minutes. Argon used as inhibitor during transition and expansion periods.

AUXIN REQUIREMENT AFTER PRETREATMENT: Need auxin be present during the expansion phase or is its role completed during the pretreatment period? This may be determined by the use of antiauxins as inhibitors. If the presence of auxin is required during the period of expansion, then the presence of a sufficiently high antiauxin concentration during the expansion period should suppress the residual effects due to auxin pretreatment. Antiauxin was therefore added to the transition and expansion period solutions. The IAA concentration of the pretreatment solution was decreased to 0.1 mg/l in order to make it possible to maintain as high as possible an antiauxin-auxin ratio in the tissues during the expansion period. The antiauxin 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) was used since it is known to exert a strictly competitive inhibition of auxin-induced growth in Avena coleoptile sections (7). It was applied in concentrations up to 2×10^{-4} M, which concentration is nontoxic and inhibits aerobic auxin-induced growth (with IAA at 0.1 mg/l) almost completely.

The data of table V show that the highest nontoxic antiauxin concentration (2×10^{-4} M) inhibits the residual auxin-induced growth by about 50 %. It is apparent, therefore, that the auxin action, accomplished during the pretreatment, can be partially undone by the presence of antiauxin during the expansion period. Auxin must evidently be present in the tissue in some form produced aerobically in order for the residual effect to persist.

The antiauxin 2,4,6-T is able to exert its effect during the transition and expansion period even under anaerobic conditions as is shown by the data of table V. Thus antiauxin can inhibit the residual effect of auxin under conditions in which added auxin is itself ineffective.

EFFECT OF METABOLIC INHIBITORS OTHER THAN ARGON: Respiratory metabolism is necessary for auxin action, and inhibitors of such metabolism other than anaerobiosis might therefore be investigated for abil-

TABLE V

INHIBITION OF RESIDUAL AUXIN EFFECT PRODUCED BY VARIOUS ANTIAUXIN CONCENTRATIONS DURING THE EXPANSION PERIOD TREATMENT

CONC OF 2,4,6-T	ΔL MM	MEDIUM OF EXPANSION PERIOD	RESIDUAL AUXIN EFFECT
None	+0.09	Air	+
2×10^{-4} M	+0.05	Air	ca 50 %
None	+0.11	Argon	+
2×10^{-4} M	+0.05	Argon	ca 50 %

Antiauxin used—2,4,6-T. Pretreatment with IAA, 0.1 mg/l, in 0.3 M mannitol for 45 min.

TABLE VI

EFFECT OF THE PRESENCE OF VARIOUS INHIBITORS DURING THE TRANSITION AND EXPANSION PERIODS ON THE RESIDUAL AUXIN EFFECT

INHIBITOR	ΔL MM	DAMAGE BY TREATMENT	RESIDUAL AUXIN EFFECT
KCN, 1×10^{-4} M	+0.07	...	+
KCN, 3×10^{-4} M	−0.04	...	−
DNP, 2 mg/l	+0.14	...	+
DNP, 4.5 mg/l	+0.03	...	−
DNP, 8 mg/l	...	+ (Dead)	
PCMB, 3×10^{-4} M	+0.07	...	+
PCMB, 5×10^{-4} M	−0.02	...	−
PCMB, 6×10^{-4} M	...	+ (Dead)	
Temp, 4° C	−0.02	...	−

Pretreatment with IAA (5 mg/l in 0.3 M mannitol for 45 min) in all cases.

ity to prevent auxin-induced growth during the expansion period. Three inhibitors with different modes of action were selected: KCN, 2,4-dinitrophenol (DNP), and *p*-chloromercuribenzoate (PCMB).

If KCN in a concentration of 3×10^{-4} M is used as the inhibitor, during the expansion period the auxin pretreated sections expand no more than do the control sections (table VI). The sections are alive and capable of response to auxin if removed from KCN at the end of the experiment. If KCN is supplied at a concentration of 10^{-4} M, the auxin treated sections expand appreciably more than do the control sections. This is due to the fact that KCN at a concentration of 10^{-4} M is only slightly inhibitory to auxin-induced growth (3). The sections are able to grow during the expansion period since they contain auxin taken up during the pretreatment. The important fact is that it is possible to select a KCN concentration which is non-toxic (over the time period used) and which completely eliminates any residual effect of the auxin pretreatment.

The inhibitor DNP, like KCN, is capable in appropriate concentration (4.5 mg/l) of suppressing any residual effect of the auxin pretreatment (table VI).

In the case of PCMB it was found necessary to decrease the buffer concentration to 0.001 M (with an accompanying rise in pH of the medium from 4.8 to 6.4) in order to get sufficient PCMB into solution to affect inhibition. Under these conditions, however, a PCMB concentration of 5×10^{-4} M does completely inhibit any residual auxin effect (table VI).

It is apparent, then, that a variety of metabolic inhibitors are able to completely block the action of auxin. Under these conditions of inhibition, no residual effect of auxin on elongation can be detected.

Low temperature was also used as an inhibitor of auxin action since it is known that auxin-induced section growth at, for example, 4° C is slow (2). After an auxin pretreatment at 25° C, sections were subjected to transition and expansion period treatments at 4° C. The data of table VI show that when low temperature is used as the inhibitor of auxin action, the auxin treated sections do not expand more than the controls over the experimental period of 90 minutes. It may be concluded that the expression of the residual auxin effect is greatly delayed at low temperature. This result is not in accord with the reports of Heyn (4) and Heyn and Van Overbeek (5) who found some residual auxin effect at 1° C, at least over periods of 2.5 hours.

DISCUSSION

It has been shown above that treatment of Avena coleoptile tissue with auxin results in changes in the tissue which can be expressed as subsequent expansion. The experimental conditions of these experiments are such that no expansion can take place during the period of auxin-action and no auxin-action can take place during the subsequent period of expansion. This eliminates the possibility that during the pretreatment period, auxin might cause an increase in cell volume by some sort of active water uptake with a resultant rupturing of cell wall bonds and a loosening of the cell wall. The lack of indication of active water uptake is in agreement with the conclusions of Ordin (6) that the movement of water into Avena coleoptile cells is entirely a passive process. Ordin has also shown that there is no detectable effect of auxin upon the osmotic concentration of the cell. The present experimental results are, however, compatible with the supposition that auxin, by acting upon the cell wall, causes a relaxation of cell wall pressure. As a result of such relaxation, water will then be taken up osmotically. The auxin-induced relaxation of cell wall pressure can take place in the absence of expansion, and, under proper conditions, can be conserved and expressed as subsequent expansion. Thimann (10) has found a similar conservation of expansion potentiality in studies on the water uptake of potato discs.

The conclusion that auxin causes a loosening of the cell wall was first proposed by A. N. J. Heyn in 1931 on the basis of experiments similar in principle to the present ones. In Heyn's and other early experiments, however, a varying amount of auxin-induced cellular expansion was allowed to take place during the auxin pretreatment which was given in a saturated atmosphere but in the absence of liquid water. Active water uptake as the cause of cell wall loosening was thus not excluded. The separation of auxin-action from expansion in the present experiments eliminates this possibility.

The present data also support Heyn's suggestion that a second process, a cell wall stiffening one, goes on in the coleoptile. This process is not the reverse of auxin-induced loosening but appears to be different and independent of the latter.

Since the period of auxin action can be separated from the act of expansion, the cell wall effect can evidently persist over at least short periods of time in the absence of further oxidative metabolism. The work with metabolic inhibitors shows, however, that for the effect to persist, a particular kind of metabo-

lism is necessary. The processes which conserve the auxin-induced cell wall expansibility are abolished by KCN, DNP, or PCMB. The present evidence suggests that low temperature also abolishes the conserving process. Heyn found, on the other hand, that auxin treated coleoptiles expanded more than non-auxin treated ones when placed in water at 1° C.

The present work suggests, then, that auxin, through some process dependent upon aerobic metabolism, causes a loosening of the cell wall and that this loosening persists until used up by expansion or counteracted by cell wall stiffening. Following the initiation of cell wall loosening in which auxin and oxidative metabolism are required, there is a period in which the alteration in cell wall may be kept alive anaerobically whether or not expansion takes place.

The magnitude of the incremental expansion which results from the auxin pretreatment is about 0.11 mm/section × hour of auxin treatment. Sections grown aerobically in the presence of auxin under similar conditions expand at the rate of about 0.15 mm/section × hour (above the no auxin control rate). The induced expansion elicited by auxin pretreatment, therefore, is a substantial fraction of the expansion which takes place simultaneously with auxin action and which is called growth. Auxin-induced growth, then, may be thought of as a continuing process of simultaneous cell wall loosenings and expansions.

Is the presence of auxin necessary during the expansion phase? The fact that the addition of 2,4,6-T during the expansion period causes an inhibition of expansion in both air and argon indicates that in order for the cell wall effect to persist, auxin must remain in the cell in some form in which it can be exchanged for antiauxin. But the fact must be explained that antiauxin supplied anaerobically is effective in inhibiting expansion due to aerobic auxin pretreatment while auxin is unable to promote expansion under the same circumstances.

Summary

1. It is shown that it is possible to completely separate in time the action of auxin on the Avena coleoptile section from the act of expansion of this tissue.

2. This conclusion is based on experiments in which auxin is added to Avena coleoptile sections which are prevented from expanding by the presence of 0.3 M mannitol. The auxin pretreatment results in changes in the section which lead to subsequent expansion under conditions in which the action of auxin is blocked by a suitable inhibitor.

3. The evidence suggests that the initial step in cellular expansion may be an auxin-induced loosening of the cell wall which allows the cell to take up water and expand osmotically.

4. The auxin-induced cell wall loosening takes place only under aerobic conditions, although the effect may persist anaerobically. The cell wall loosening effect does not persist in the presence of KCN, dinitrophenol, or *p*-chloromercuribenzoate.

5. The continued presence of auxin seems to be required for the persistence of the cell wall loosening effect. Addition of an antiauxin during the expansion period partially inhibits the expansion which would otherwise take place.

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EXPERIMENTS ON THE BIOGENESIS OF THE PYRIDINE RING IN HIGHER PLANTS [1,2]

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That tryptophan may be a precursor of nicotinic acid in rats and Neurospora was shown in the former by feeding experiments with niacine-deficient animals (13) and in the latter by studies with mutants requiring 3-hydroxyanthranilic acid (1), a metabolite known to arise from tryptophan. The mammalian experiments have been substantiated with isotopic experiments (8, 7) where tryptophan-3-C^{14} and 3-hydroxyanthranilic-7-C^{14} acid have resulted in the excretion of N-methylnicotinamide-7-C^{14}.

Equivalent experiments with higher plants do not provide similar results. For example, in excised peas, where a steady state of nicotinic acid exists, so that precursors of nicotinic acid accumulate as trigonelline (N-methylnicotinic acid), no accumulation of the latter occurred when the plants were fed ornithine, tryptophan, citrulline or pyridoxine (22). More directly, the trigonelline from garden peas fed trytophan-3-C^{14} was inactive (11). We have also synthesized 3-hydroxyanthranilic acid-7-C^{14} and fed them to excised soybean leaves. The lack of conversion of this compound to trigonelline is reported here.

Grafting experiments by Dawson (2) have shown that nicotine [β-(α-N-methylpyrrolidyl)-pyridine] is synthesized solely by the roots of the tobacco plant, while its isomer, anabasine [β-(α-piperidyl)-pyridine] is synthesized both in roots and leaves. Independent studies (3, 9) have shown that the pyrrolidine ring of nicotine may be synthesized from ornithine via a symmetrical precursor (e.g., putrescine or pyrrolidine), which, however, contributes no activity to the pyridine portion of the ring. The conversion of lysine to pipecolic acid occurs in both plants and animals (6, 12, 18). However lysine is not converted to the pyridine portion of nicotine (10) nor, as will be shown below, is it utilized for anabasine biogenesis. A similar situation exists with regard to hydroxylysine. Anabasine made by photosynthesis with $C^{14}O_2$ is uniformly labeled.

METHODS

Excised leaves of *Nicotiana glauca* were used for experiments with anabasine. Petioles of the leaves were placed in small test tubes containing the solution being assimilated. An average leaf of this plant transpires approximately 2 ml/hr. Since this requires five to six replenishments of the available volume of solution in the tube, essentially quantitative uptake is assured. In $C^{14}O_2$ assimilation, the leaves were permitted to photosynthesize until > 95 % of the atmospheric radioactivity was utilized (about 3 hrs). Following both types of feeding the excised leaves were then permitted to sit in the laboratory in tap water for 48 hours, following which they were extracted with 80 % aqueous ethanol, lipoidal pigments extracted with petroleum ether, the solution made alkaline (pH about 10 to 11) and the alkaloids extracted with diethyl ether. Anabasine was obtained chromatographically (20) (racemization occurs, as well as partial separation of the racemates) and purified by rechromatography. Part of the anabasine was characterized by conversion to its dipicrate, the remainder oxidized to nicotinic acid with permanganate (17). The nicotinic acid was purified by sublimation and a portion decarboxylated (450 ° C) to CO_2 and pyridine (3), the latter being caught in a solution of picric acid. The CO_2 is about 20 % contaminated with CO_2 from the partial degradation of pyridine.

Hawkeye soybeans, grown in a greenhouse under conditions of vegetative photoperiod were found to be a satisfactory source of trigonelline. The concentrated 80 % aqueous ethanol leaf extract was ion-exchanged on IR-120. The 2 *N* NH_4OH eluate, combined with a subsequent water wash, was discarded; trigonelline was then removed with 5 % diethylamine in water and run descendingly in Munier's (14) solvent, until the nicotinic acid ($R_f = 0.08$) was almost to the edge, the trigonelline ($R_f = 0.02$) then being about one-fourth of the way down. The trigonelline area (made visible with Draggendorf's reagent) was eluted with hot water, followed by ethanol, and repeated with hot water. The entire extract was banded on a strip for paper electrophoresis, along with trigonelline and 3-hydroxyanthranilic acid reference spots. When run at pH = 8.6 (0.1 M citrate buffer) and 800 v (about 0.5 ma), the trigonelline moves cathodically about 1.5 cm, while the 3-hydroxyanthranilic acid moves anodically about 5.0 cm.

The synthesis of 3-hydroxybenzoic-7-C^{14} acid was accomplished by known procedures: benzoic acid → sodium *m*-sulfobenzoic acid (16, 19) → *m*-hydroxybenzoic acid → *m*-methoxybenzoic acid (5) → 2-nitro-3-methoxybenzoic acid (15) → 2-amino-3-hydroxybenzoic acid. The final yield was 580 mg 3-hydroxyanthranilic-7-C^{14} acid from an initial 5 gm benzoic-7-C^{14} containing 1 mc C^{14}. The last step is primarily responsible for the poor yield.

The 3-hydroxyanthranilic acid (3-HAA) is difficultly soluble in water. It oxidizes slowly in neutral solutions, and rapidly in alkali. Three different solutions were used to feed excised leaves: 1) One mg 3-HAA suspended in 5 ml H_2O. To 0.5 ml were added one drop conc NH_4OH and several drops ethanol. The solution was concentrated in vacuo to 0.14 ml, of which 0.13 was fed to the excised leaf. A

[1] Received March 27, 1956.

[2] The author gratefully acknowledges the partial support of this program by the National Science Foundation.

similar experiment with 4 mg of 3-HAA in 0.5 ml H_2O plus 2 drops NH_4OH resulted in a brownish solution which was still fluorescent in UV. The absorption of this solution by four leaves, resulted in no obvious ill effects to the leaves after 3.5 days in tap water in the green house. 2) A solution of 4 mg 3-HAA required about 3 ml of 0.1 M pH 5.0 phosphate buffer. Uptake by the leaf was not too satisfactory. 3) Similarly, a solution of 4 mg of 3-HAA required about 3 ml of 0.1 M citrate buffer, pH 4.35. Although there was no browning, the large amount of citrate appeared somewhat deleterious to the plant, since proper turgor was never reestablished despite occasional petiole cutting to unplug xylem vessels.

Results and Discussion

The uniformity of distribution of radioactivity in anabasine is shown in table I, assuming biogenetic equivalence of the carbons in each of the rings. With lysine-2-C^{14} (obtained from Tracerlab, Boston Massachusetts) and an initial activity in the 80 % ethanol extract of 2.1×10^5 cpm, the radioactivity in anabasine, though positive, was so small as to be neglected.

Hydroxylysine-6-C^{14} (0.4 μc) [3] resulted in an initially very low, but active anabasine dipicrate which successive recrystallizations showed to be active primarily as a result of contamination: initial activity, 2.16 cpm/mg; 1st recrystallization, 0.84 cpm/mg; 2nd recrystallization 0.28 cpm/mg. On the other hand, glucose-U-C^{14} (prepared photosynthetically), resulted in respective activities of 7.59, 6.71, and 6.44 cpm/mg, in a total yield of about 0.5 %, based upon the activity of the 80 % ethanol extract. The degradation of the anabasine from glucose-U-C^{14} is in progress.

Radiotrigonelline could not be found either from $C^{14}O_2$ photosynthesis or from administration of 3-HAA-7-C^{14}.

The experiments with 3-HAA are not entirely satisfactory. Certainly a specific activity greater than 0.2 μc/mg would be desirable—although considering the uniqueness of the reaction, this should be ample. More perturbing is the question of oxidation of the compound to an unusable derivative. This can be answered only by stating that radioactive material, chromatographically identical with 3-HAA, could still be extracted from leaves fed 3-HAA within less than 24 hours after feeding. The possibility exists that the carboxyl group (and consequently the radioactivity) of 3-HAA is lost by exchange, or some other process, during transformation of 3-HAA to nicotinic acid. However, in the light of the parallel mammalian experiments, this possibility must be rather remote. The experiments with $C^{14}O_2$ are quite straightforward. In soybeans the ultimate precursors of trigonelline must be formed in the roots—unless excision so alters the metabolism that the leaf is incapable of its biosynthesis. Direct experiments with tryptophan-3-C^{14} are in progress.

Considering the ease of conversion of lysine to pipecolic acid, it is surprising that the former, or hydroxylysine, is not a precursor of the piperidyl ring. The small amounts of activity found in anabasine when these substrates are fed may be real; however, they are obviously not direct since the yield is far below that obtained by photosynthesis or with the feeding of glucose-U-C^{14}. Consequently the origin of both the piperidyl as well as the pyridyl ring of anabasine is still unknown. The possibility of the transformation of γ-methylene glutamine (which is known to occur in higher plants [21, 4]) to the pyridyl ring is being considered. This is, in a sense, the reversal of the known alkaline degradation of nicotinic acid to α-hydroxymethyl glutaric acid, which, on vacuum distillation, is converted to the corresponding methylene.

R. F. Dawson, et al (Jour. Amer. Chem. Soc. 78: 2645–2646. 1956) have shown that nicotinic acid, with tritium substituted in the ring, is incorporated without loss of specific activity into tobacco root nicotine. It is suggested that nicotinic acid may also serve as the precursor of the pyridine ring of anabasine and other pyridine-containing compounds. While this experiment does not resolve the problem of the mechanisms of the formation of the pyridine ring per se, it suggests that when the origin of nicotinic acid in higher plants is known, we will simultaneously have delineated the biogenesis of many naturally-occurring pyridines.

Table I

Degradation of Anabasine-C^{14}

Substance	Specific activity cpm/mgc*
Anabasine (picrate)	99.2
Nicotinic acid	100.0
Pyridine (picrate)	97.5

* Cpm/mgc = counts per minute per mg carbon of the pertinent portion of the molecule.
Standard error < 3 %.

[3] One microcurie was obtained through the generosity of Drs. M. Sarett and D. D. Van Slyke, Brookhaven National Laboratory.

Summary

1. Anabasine [β-(α-piperidyl)-pyridine] formed during photosynthesis in $C^{14}O_2$ is uniformly labeled.

2. Glucose-U-C^{14} may be used as an ultimate precursor for anabasine.

3. Neither lysine-2-C^{14} or hydroxylysine-6-C^{14} are definitive precursors for anabasine.

4. Radiotrigonelline (N-methylnicotinic acid) is not formed during photosynthesis of excised soybean leaves in $C^{14}O_2$.

5. 3-Hydroxyanthranilic-7-C^{14} acid does not appear to be a precursor of trigonelline in the soybean leaves.

The writer wishes to acknowledge the skillful assistance of Messrs. D. Maier, J. Wickliff, and J. Hanson in various portions of this study.

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THE INFLUENCE OF SALTS ON THE ACTIVITY OF PARTICULATE CYTOCHROME OXIDASE FROM ROOTS OF HIGHER PLANTS[1,2]

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Recent investigations by Webster (21) have shown that cytochrome oxidase is widely distributed in higher plant species and it has been suggested (7) that this enzyme may play a major role in the terminal transfer of electrons in plant respiration. Lundegårdh (13) reported that the absorption spectrum of bundles of roots from wheat and other cereals revealed the existence of a complete cytochrome oxidase which was similar to that observed in animal tissues and many microorganisms. He calculated that this system was responsible for 50 to 75 % of the total aerobic respiration. Fritz and Beevers (6) observed that extracts from etiolated pea, wheat and barley seedlings contained cytochrome oxidase at all stages of development. When the amount of enzyme in the tissues was evaluated in terms of activity at an infinite concentration of cytochrome c, it was concluded that shoots or roots of peas, and shoots of either barley or wheat contained sufficient amount of the enzyme to account adequately for the respiration of these tissues. The roots of barley and wheat also contained the enzyme but the amounts extracted were not capable of mediating all the respiratory oxygen absorption. The results with wheat were in good agreement with those reported by Lundegårdh. It would appear that the importance of cytochrome oxidase in many higher plant species may approach that of this enzyme in the respiration of animals and aerobic microorganisms.

Several investigators have observed that salts

[1] Received April 3, 1956.

[2] Contribution from the Division of Biological Sciences, North Carolina Agricultural Experiment Station and published with the approval of the Director as paper No. 725. This research was supported in part by a grant from the Chilean Nitrate Educational Bureau.

stimulated the activity of cytochrome oxidase preparations from animal tissues. According to Smith (17) the activity of the enzyme in particulate fractions of kidney or heart was markedly increased by salt. The maximum effect was obtained with NaCl at a concentration of 0.11 M. A similar observation was made by Riley (16) who reported that a concentration of 0.1 M NaCl increased the activity of a cytochrome oxidase preparation from mouse melanomas by 40 %. Megumu (15) has reported that univalent cation salts at a concentration of 0.05 M maximally increased the activity of cytochrome oxidase from ox heart muscle. Divalent cation salts were stimulatory at lower concentrations.

There are relatively few experiments reported in the literature concerning the influence of salt on the activity of cytochrome oxidase from higher plant tissues. Lundegårdh (12) added macerated wheat roots to a solution containing reduced cytochrome c and observed that 41 % of it was oxidized in five minutes. In a comparable experiment where the solution contained KNO_3 or KCl at a concentration of 0.1 M, 73 % of the reduced cytochrome c was oxidized in a 5-minute period. Weinstein et al (22) observed the greatest cytochrome oxidase activity of a homogenate of sunflower leaves in a phosphate buffer (mixture of Na_2HPO_4 and KH_2PO_4) at pH 6.0 and at a concentration of approximately 0.13 M. The activity was markedly depressed with buffer concentrations greater or less than this value. No attempt was made to distinguish between the effect of anions and cations or to determine whether or not an osmotic phenomenon was involved.

It has been well established (14) that the addition of salts to plant tissues containing low salt contents markedly increased their respiratory rates. This increased respiration is sensitive to heavy metal inhibitors and carbon monoxide inhibition is reversed by light indicating the involvement of cytochrome oxidase. In view of the possible significance of these observations in an understanding of the mechanism of the salt accumulation and of the brief reports that the activity of plant cytochrome oxidase per se was influenced by salts, the experiments reported here were initiated in an effort to provide more detailed information on the influence of salts on cytochrome oxidase from plant sources. The experimental evidence also may have an important bearing on the role of certain cations in plant metabolism.

Materials and Methods

Preparations: Particulate preparations used in the various experiments were made from the roots of soybean (*Glycine Max* Merr.), tobacco (*Nicotiana Tabacum* L.), oat (*Avena sativa* L.) or spinach (*Spinacia oleracea* L.) plants which were grown in flats of sand in the greenhouse. The age of the seedlings ranged from 6 to 20 days. Extracts of root tissue were prepared as outlined by Webster (21) with the exception that the isolation medium contained 0.3 M sucrose and 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer instead of phosphate buffer. In the preparation of the extracts 1 gm of roots was ground in a cold mortar with 20 ml of a cold solution of buffer and sucrose, then homogenized for 1 minute with a Ten Broeck homogenizer and centrifuged at $1000 \times g$ for 15 minutes. The supernatant was decanted and centrifuged at $20{,}000 \times g$ for 15 minutes. The particles were collected and washed with 10 ml of the sucrose-buffer solution then suspended in a volume of sucrose-buffer equivalent to one fifth the original volume of the homogenate. Extracts prepared by this procedure were used in all experiments except that reported in figure 4. In this experiment (fig 4) the extract was prepared as described above, with the exception that buffer was omitted from the isolation medium. Also the suspension of particles used in this experiment was dialyzed over night in 2 liters of 0.3 M sucrose in an effort to remove endogenous salts.

A stock solution of 12 mg/ml of cytochrome c (Sigma Chemical Company of St. Louis, Missouri) in 0.05 M Tris buffer at pH 7.0 was chemically reduced with sodium hydrosulfite (3) and thoroughly aerated before use. The concentration of the stock solution was determined by use of the difference in molar extinction coefficients of oxidized and reduced cytochrome c at 550 mμ which is 1.96×10^4 (8). The stock solution of cytochrome c used in the experiment reported in figure 4 and the extract used in this experiment were dialyzed overnight against a 0.3 M sucrose solution before they were used. After dialysis the reduced cytochrome c concentration was determined by the spectrophotometric procedure. Solutions of reduced cytochrome c were relatively stable when stored at $-10°$ C.

Tris salts of Cl^- and phosphate (mixture of $H_2PO_4^-$ and $HPO_4^=$) used in the experiments reported in figure 3 were prepared by adding sufficient amounts of appropriate acids to Tris base to adjust the pH to 7.0. Tris buffer used in the various procedures was prepared by neutralization with HCl until the desired pH value was obtained. The solution of $NaHCO_3$ used in the experiments reported in figure 2 was adjusted to pH 7.5 by bubbling CO_2 into the solution.

In the experiment involving purification of the enzyme extract with ion resins, a mixture of hydroxyl saturated anion resin IRA-400 and hydrogen saturated cation resin IR-120 (both obtained from Rohm and Haas Company, Philadelphia) were mixed together in proportions that resulted in a pH of 6.5 in distilled water. The enzyme was dialyzed for 12 hours against a suspension containing 5 gm of mixed resins in 2 liters of cold distilled water.

Standard Assay: Enzyme activities were assayed spectrophotometrically at 550 mμ with a Beckman DU spectrophotometer (3). The reaction mixture in a final volume of 1 ml contained the following constituents in micromoles: 25 Tris buffer at pH 7.0, 0.051 reduced cytochrome c, desired concentration and type of salt, and enzyme containing 0.1 to 0.2 mg protein. Enzyme activity is indicated as the decrease in optical density per mg protein between 15 and 75

seconds after the reaction was started by the addition of the enzyme. Reduced cytochrome c was not oxidized when boiled enzyme was used in the assay procedure with or without the various experimental additions to the mixture. Under the conditions of the experiments the oxidation rates remained linear for at least two minutes which is in agreement with the observations of Fritz and Beevers (6). The mixture was stirred prior to each reading in order to prevent errors due to the settling of particles in the cuvette. The various experiments were run three times and the results averaged. Maximum deviation from the mean was 8 % but in the majority of experiments the deviation was much less than this.

Other Methods: Aliquots of purified assay mixtures were ashed in a muffle furnace at 600° C and analyzed for Na and K by use of a Perkin-Elmer flame photometer (23). Protein content of extracts was estimated by use of either Folin's phenol reagent (11) or by total N analyses (20). Results with the different methods were in good agreement.

Experimental Results

Effects of Various Cations: Extracts of roots from four plant species were prepared and the effect on the activity of cytochrome oxidase of chloride salts at varying concentrations observed. In figure 1 A the influence of chloride salts on cytochrome oxidase activity of extracts from soybean roots is shown. Additions of univalent cation salts resulted in a striking increase in activity. A maximum stimulatory effect was exhibited at a concentration near 0.1 *N* and greater concentrations were inhibitory. It is of interest to note that chlorides of Na^+ and Li^+ at optimum concentrations activated less than chlorides of K^+ and NH_4^+. The degree of activation at optimum univalent salt concentration in this particular experiment was greatest with the least hydrated cations possessing greatest relative mobilities (9). Divalent cation chlorides activated slightly up to 0.05 *N*, but increased concentrations caused a sharp decline in activity resulting in almost complete inhibition at 0.2 *N*. $CaCl_2$ at concentrations below 0.05 *N* stimulated enzyme activity more than $MgCl_2$ at comparable concentrations. At all except low concentrations, univalent cation salts were more effective than the divalent ones. The influence of nitrate salts of Mg^{++}, Ca^{++}, K^+ and Na^+ on cytochrome oxidase activity of soybean extract was similar to that observed with the respective chloride salts (table I). These results were not included in figure 1 because the effect of the two anions were so similar that an overlapping of many curves would have resulted. The data in this table also show that various concentrations of sucrose had little effect on the activity of the enzyme.

Table I

Effect of Various Anions and Cations on the Activity of Cytochrome Oxidase from an Extract of Soybean Roots *

Additions	Conc of added salt (*N*) or sucrose (M)			
	0.00	0.05	0.10	0.20
	– O.D. change per min per mg protein			
Various anions				
NaCl	0.23	0.37	0.65	0.45
NaBr	0.23	0.35	0.70	0.40
NaI	0.23	0.38	0.65	0.43
Na_3 citrate	0.23	0.40	0.70	0.45
Various cations or sucrose				
$NaNO_3$	0.23	0.40	0.68	0.40
KNO_3	0.23	0.40	0.70	0.45
$Ca(NO_3)_2$	0.23	0.42	0.12	0.05
$Mg(NO_3)_2$	0.23	0.40	0.18	0.05
Sucrose	0.23	0.20	0.18	0.15

* The standard assay procedure was used with the addition of salts or sucrose as indicated. One tenth ml of soybean root extract containing 1.6 mg protein per ml was used as the enzyme.

Table II

Effect of Combinations of Salts on Cytochrome Oxidase Activity from an Extract of Soybean Roots *

Salts	Total salt conc (*N*)					
	0.00	0.05	0.075	0.10	0.15	0.20
	– O.D. change per min per mg protein					
NaCl	0.33	0.52	0.73	0.77	0.67	0.37
KCl	0.33	0.55	0.70	0.79	0.67	0.40
½ KCl + ½ NaCl	0.33	0.47	0.70	0.80	0.67	0.37
$CaCl_2$	0.33	0.50	0.30	0.20	0.10	0.08
½ CaCl + ½ KCl	0.33	0.50	0.45	0.35	0.15	0.13

* The standard assay procedure was used with the addition of salts as indicated. The assay included 0.10 ml soybean extract containing 1.4 mg protein per ml.

The effect of chloride salts of various cations on the activity of cytochrome oxidase from roots of tobacco, spinach and oat is shown in figures 1 B, 1 C and 1 D and the results are generally comparable with those obtained with soybean. In all experiments with the exception of those where extracts of oat roots were used, KCl at optimum concentrations stimulated enzyme activity slightly more than NaCl. The effect of divalent cation salts on cytochrome oxidase activity from oat and spinach extracts was similar to that observed with soybean, but with extracts from tobacco, $MgCl_2$ was considerably more effective than $CaCl_2$ at low as well as at high concentrations. This is particularly noticeable at the concentration of 0.05 *N*.

It is apparent from figure 1 that univalent cation salts have similar effects on enzyme activity. The activating effect of combinations of salts as compared with single salts is shown in table II. A mixture containing equal concentrations of NaCl and KCl behaved in a manner similar to the same total concentration of either of the single salts indicating an additive effect of univalent cation salts. The activation of cytochrome oxidase by an equal mixture (N) of $CaCl_2$ and KCl was similar to that noted with the single

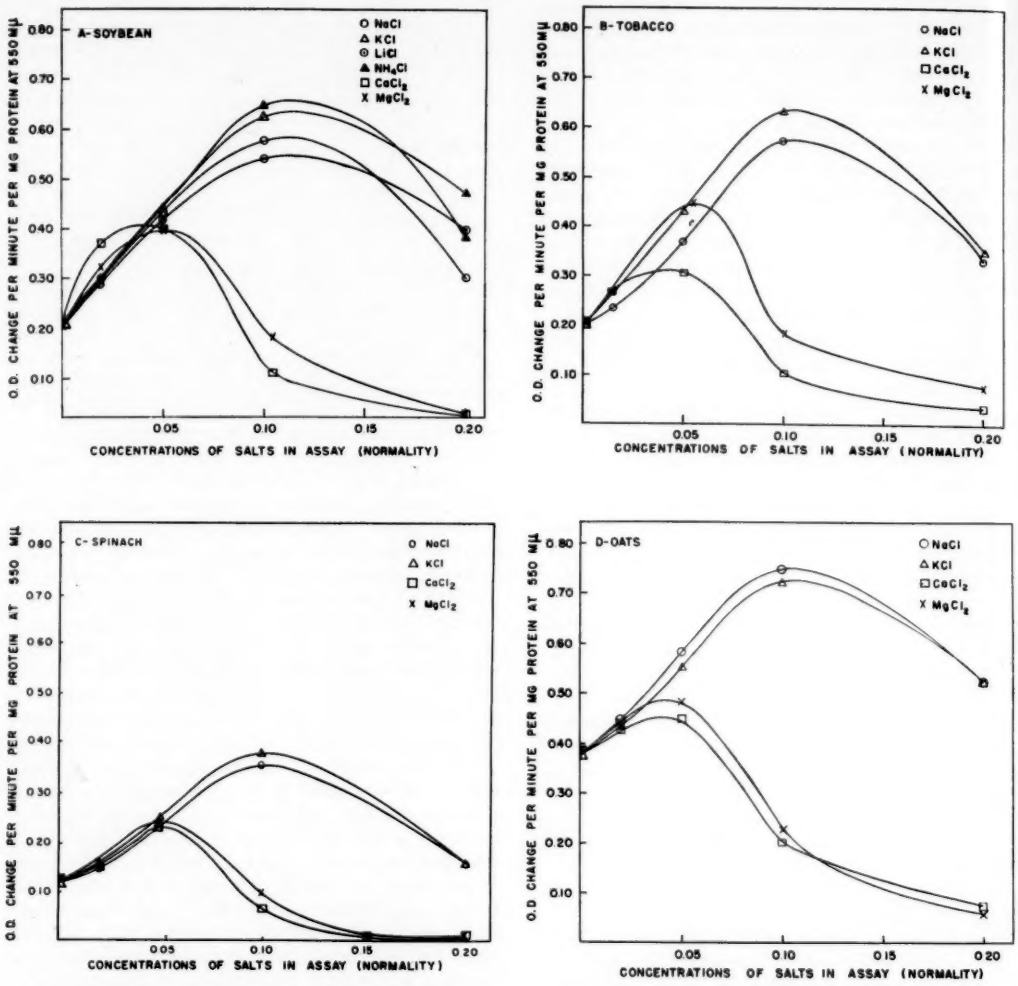


FIG. 1. Effect of various concentrations of salts on the cytochrome oxidase activity of extracts from roots of several plant species. Procedure was that of standard assay with variations in concentration and type of salt as indicated. The enzyme preparations were from the following species: A—0.10 ml soybean extract containing 1.2 mg protein per ml; B—0.10 ml tobacco etxract containing 1.3 mg protein per ml; C—0.10 ml spinach extract containing 1.7 mg protein per ml; D—0.10 ml oat extract containing 1.4 mg protein per ml.

In this figure and the subsequent figures the O.D. changes are negative.

salts up to a total concentration of 0.05 *N*. At greater concentrations an interaction between Ca^{++} and K^{+} salts was apparent as indicated by the difference in enzyme activities obtained from combinations of salts as compared with single salts. Where Ca^{++} was at maximum stimulatory level, the addition of univalent cation salts suppressed activity.

EFFECT OF VARIOUS ANIONS: Univalent and divalent cation salts differ in their influence on cytochrome oxidase activity but the effect is not independent of the anion present. It can be seen from figure 2 that sodium salts of Cl^{-}, NO_3^{-}, and $SO_4^{=}$ were equally effective in activation of the enzyme. The sodium salts of Br^{-}, I^{-} and citrate (tri-sodium) influenced the activity in a manner similar to the chlorides as shown in table I. From figure 2 it is apparent that the addition of phosphate ($HPO_4^{=} + H_2PO_4^{-}$) increased the activity of the enzyme from soybean roots to a greater degree than other anions. The increased stimulatory effect of phosphate also was observed with extracts of spinach, oat and tobacco roots. The stimulation from phosphate salt at most concentrations was approximately 20 % higher than that of other salts. The effect of phosphate was manifested at con-

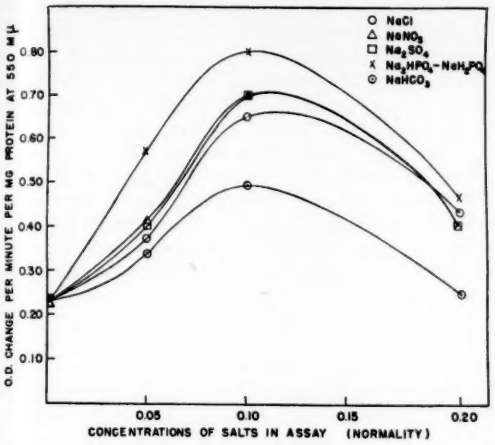
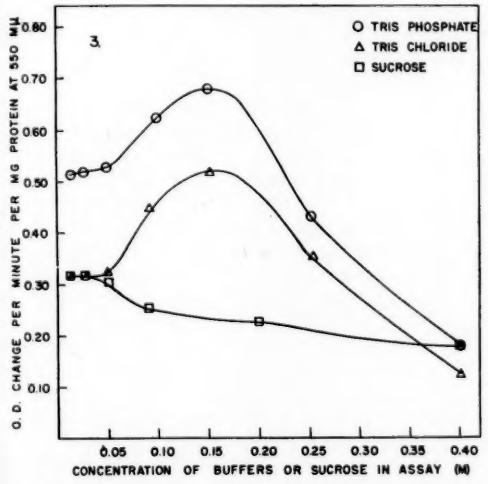


FIG. 2. The effect of various concentrations of sodium salts on the activity of cytochrome oxidase from soybean roots. The procedure of standard assay was followed with the exception that all determinations were made in 0.025 M Tris buffer at pH 7.5. The assay mixtures contained variable concentration and kinds of salts as indicated. The preparation of the bicarbonate solution is described in Materials and Methods. Assays included 0.1 ml soybean extract containing 1.7 mg protein per ml.

centrations as low as 0.0005 M. Bicarbonate salts at the same pH and cation concentration as the above mentioned salts resulted in little activation at concentrations that were optimum for other salts and pronounced inhibition at high concentrations. This inhibition was noted with extracts of various plant species.

REQUIREMENT OF SALT FOR ACTIVITY: In the experiments where the effect of various cations and anions on the activity of cytochrome oxidase was studied, marked stimulation was noted from added salts. Since all these assay mixtures contained 0.025 M Tris chloride as a buffer, it was deemed necessary to test the influence of various concentrations of buffer on the enzyme activity. The results of such an exeriment are reported in figure 3. Tris salts of Cl^- and phosphate (mixture of $H_2PO_4^-$ and $HPO_4^=$) activated the enzyme in a manner similar to other univalent cation salts. The difference in stimulation between chloride and phosphate salts was observed again and indicated a specific action of the phosphate ion. Tris phosphate at concentrations of 0.0125 M resulted in marked increases in activity when compared with Tris chloride at the same concentrations.

An experiment was set up to determine enzyme activity in an assay mixture containing no added Tris or inorganic salts. The preparation of materials is described in Materials and Methods. Enzyme activ-



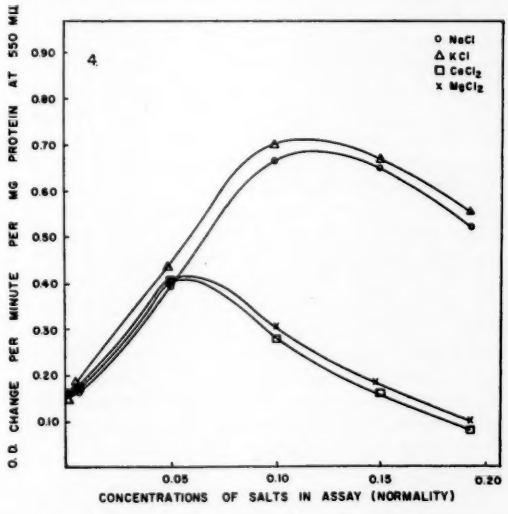


FIG. 3. Effect of various concentrations of Tris salts on the activity of cytochrome oxidase from soybean roots. The standard assay procedure was used with variation in buffers (preparation of Tris phosphate and Tris chloride is described in Materials and Methods) as indicated. Extracts were prepared in either Tris chloride or Tris phosphate depending on which buffer was used in the assays. Assays included 0.1 ml soybean extract containing 1.1 mg protein per ml.

FIG. 4. The influence of salts on activity of cytochrome oxidase from soybean roots in a purified medium (see Materials and Methods). The assay mixture in a final volume of 1 ml contained 0.051 micromoles of dialyzed reduced cytochrome c; 0.1 ml dialyzed extract containing 1.6 mg protein per ml, and the concentration and types of salts indicated. No buffer was used in the assay mixtures and the pH varied between 5.2 and 5.3 during the determinations. All salt solutions were adjusted to pH 5.2 before use.

ity was present in this purified medium as shown in figure 4, and the salt stimulation was similar to previous experiments where buffers were used. An analysis of a sample of the entire purified reaction medium showed that concentrations of 1.5×10^{-4} M potassium and 7.5×10^{-5} M sodium were present in the mixture.

In another experiment, dialysis of the enzyme against a mixed resin, as described in Materials and Methods, failed to decrease the cytochrome oxidase activity in the reaction mixture containing no added salts. An analysis of the entire reaction mixture under these conditions showed 1.25×10^{-4} M potassium and 6.3×10^{-5} M sodium were present. In another attempt to remove salts, the enzyme preparation was placed for 30 minutes in a cold Gooch crucible in contact with a mixture of the anion and cation resins described in Materials and Methods. The enzyme was recovered by suction and assayed for activity but no absolute dependence on salt was observed.

Discussion

The results of these experiments clearly show that both type and concentration of salts in contact with suspensions of particulate matter from roots of various plant species profoundly influenced the cytochrome oxidase activity of the particles. When the anions were held constant as the chlorides (fig 1 and table I) the various cations up to a concentration of 0.05 *N* generally resulted in similar effects on the activity of cytochrome oxidase. When concentrations of cations greater than 0.05 M were added to assays then univalent cations continued to stimulate activity up to a concentration of 0.12 M whereas concentrations of divalent cations greater than 0.06 M were inhibitory. When the results were plotted on an ionic strength basis different effects of univalent and divalent cations were observed at concentrations greater than 0.07 *N*. The influence of salts on the activity of cytochrome oxidase from soybean roots is not independent of the anion associated with salt as indicated by the results in figure 2 and table I. The sodium salts of NO_3^-, $SO_4^=$, Cl^-, I^- and citrate$^{\equiv}$ at comparable pH values were strikingly similar in their effect on the activity. Sodium phosphate (Na_2HPO_4-NaH_2PO_4) on the other hand consistently increased activity to a greater degree than the other sodium salts. The specific inhibitory action of bicarbonate ions on cytochrome oxidase has been observed consistently with enzyme preparations from various species and is the subject of another communication.

In view of the relatively high concentrations of salts required for maximum cytochrome oxidase activity in isolated particulate matter from soybean root and the relatively low concentration of salts needed for maximum salt respiration of intact tissues (4) one might conclude that entirely different phenomena were involved in each case. These differences may be accounted for by assuming that intact tissues possess a greater accumulatory capacity for salts than the particulate preparations containing cytochrome oxidase.

It seems possible that different concentrations and types of salts may influence cytochrome oxidase activity by their effects on the physical structure of the particles. Stafford (18) has established that cytochrome oxidase in higher plant tissue occurs in intracellular particles of the size of mitochondria. Farrant et al (5) have shown with the electron microscope that mitochondria from red beet prepared in 0.45 % KCl appeared as large diffuse discs whereas those prepared in 30 % sucrose appeared as compact spheres. It has been demonstrated that the cytochrome oxidase activity of particles isolated from animal livers was strongly affected by the physical state of the particles and that salts markedly influenced the physical appearance of them (2). One might speculate that the type and concentration of the various salts used in the experiments reported in this manuscript affected the physical properties of the particles and therefore possibly altered the accessibility of the enzyme to either oxygen or reduced cytochrome c. A microscopic examination of the particles from soybean seedlings in both water and 0.1 *N* KCl indicated dimensions of mitochondrial size, but no gross differences in physical structure were observed. It is necessary to conduct more detailed studies with these particles before a definite conclusion concerning the effect of salt on gross structure can be made.

Since it has been clearly established (5) that beet mitochondria possess a membrane and that cytochrome oxidase is associated with mitochondria (18), it is necessary to consider the possibility that the various reactants in the cytochrome oxidase reaction have to penetrate a membrane during the course of the enzymatic reaction. In this event various species and concentrations of ions would very likely have differential effects on membrane permeability (22) and therefore the rates of the enzymatic reaction. Lehninger (10) has pictured an "internal" and "external" pathway of electron transport from the substrate to molecular oxygen in mitochondrial preparations. Externally added reduced cytochrome c apparently does not penetrate to the internal site of electron transport within the mitochondrial body unless some alterations are made in mitochondrial permeability or structure. Apparently it is necessary for electrons to be transported through the "internal" pathway in order to obtain oxidative phosphorylation. Information concerning the relationship of salts to the pathway of electron transport and to oxidative phosphorylation in plant mitochondria remains for future study.

In an effort to explain the influence of salts on the oxidation-reduction balance of cytochromes in living wheat roots, Lundegårdh (14) proposed that if two molecules capable of undergoing oxidation-reduction were separated from each other, apparently in some structural unit, they may fail to react unless salts are present in the surrounding medium. In this proposal the movement of ions may compensate for the electrostatic potential between the reactants involved in the oxidation-reduction. In order to apply this generalized explanation to the cytochrome oxidase reaction it

seems that particulate cytochrome oxidase would be necessary to meet the structural requirements of a salt dependent system. If cytochrome oxidase were in true solution and in intimate contact with a solution of reduced cytochrome c it seems logical to expect that no salt influence would be manifested if the general mechanism proposed by Lundegårdh is operative.

The striking stimulatory action of univalent cation salts on the activity of cytochrome oxidase may have a bearing on the role of univalent cations in plant metabolism. It is well established that K^+ is the major cation in plant tissues and that Na^+ will partially replace K^+ in its role as a univalent cation in certain species. Since Na^+ and K^+ are approximately equally effective in the activation of cytochrome oxidase it seems that this enzyme may represent a metabolic site where replacement could occur. It is of interest to note that dry plant tissues containing 2 to 4 % K (1) would contain a concentration calculated on a fresh tissue basis of approximately 0.05 to 0.1 *N*. As already indicated this range in concentration of univalent cation salts markedly stimulated the activity of cytochrome oxidase in vitro. A significant physiological role of univalent cations other than K^+ and Na^+ in the activation of cytochrome oxidase in vivo seems unlikely in view of either the low concentrations or relative toxicities of other such cations that are normally present.

Summary

A study has been made of the influence of various concentrations and kinds of salts on the activity of cytochrome oxidase associated with particulate preparations showing characteristics of mitochondria from the roots of certain higher plant species.

The activity of the enzyme from all the species studied including soybean, tobacco, oat and spinach was markedly influenced by salts. When the anions were held constant as chlorides and the effect of cations compared on an equivalent basis, increasing concentrations of univalent cation salts stimulated activity until a maximum effect was obtained at a concentration of approximately 0.12 *N*. Concentrations greater than this resulted in a progressive decline in activity. The addition of chloride salts of divalent cations was less stimulatory than salts of univalent cations. The maximum increase in activity was obtained at a concentration of near 0.05 *N* and greater concentrations were inhibitory. The concentration of Tris chloride used as a buffer also influenced the activity of the enzyme.

Experiments were conducted with extracts from soybean roots where the cation of salts was kept constant as Na^+ and the influence of equivalent concentrations of anions studied. Cl^-, NO_3^-, $SO_4^=$, Br^-, I^- and citrate influenced the activity in a similar manner but phosphate ($H_2PO_4^- + HPO_4^=$) resulted in approximately 20 % greater activity than other anion salts. The effect of $NaHCO_3$ on the activity when compared with other anion salts at the same pH values was inhibitory.

An absolute salt requirement for cytochrome oxidase activity was not demonstrated; however, analyses of purified assay mixtures indicated the presence of small concentrations of both Na^+ and K^+.

The influence of salts on the activity of cytochrome oxidase was not an osmotic manifestation since various concentrations of sucrose in the assay medium had no appreciable effect.

The experimental results are discussed in relation to the possible mechanism of the differential action of various salts and also in relation to the possible role of certain cations in plant metabolism.

The authors gratefully acknowledge the technical assistance of Mrs. Thea Schostag.

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OVERWINTERING TRENDS OF COLD RESISTANCE AND CARBOHYDRATES IN MEDIUM RED, LADINO, AND COMMON WHITE CLOVER [1,2]

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Medium red clover, *Trifolium pratense* L., and ladino clover, *Trifolium repens* L., are used extentively in the northern areas of the United States in hay and pasture mixtures while common white clover, *Trifolium repens* L., occurs naturally in most of the closely grazed pastures. Unfortunately, these true clovers are often killed or weakened during the winter months, and maintaining stands over winter is one of the important problems related to their culture in the north. A better understanding of the overwintering physiology of these legumes is needed in order to provide information that will aid in the maintenance of stands during the winter period.

Several workers have studied the trend and development of cold resistance in alfalfa during the fall and winter months (2, 3, 5, 8, 14). Similar work with red, ladino, and common white clover is limited. Bula and Smith (2) compared the cold resistance trend in medium red clover with that in Ranger alfalfa and biennial sweetclover under Wisconsin conditions. The trends in field-grown seedling plants of alfalfa and sweetclover were similar although sweetclover developed resistance more rapidly and reached a higher level than alfalfa. Red clover developed cold resistance later, more slowly, and did not reach as high a level as alfalfa or sweetclover. Most of the resistance in these legumes was developed by the time of the first sampling following permanent freezing of the soil surface in late November to mid-December. Cold resistance in red clover and alfalfa began to decrease after mid-February while it was retained longer in sweetclover. Cold resistance was not lost rapidly until after the snow was gone and the soil surface thawed in late March. Bula et al (3) have shown further with alfalfa that certain responses differed when the same varieties were grown at diverse latitudes. Smith (15) has compared the survival of field-hardened stolons of common white and ladino clover when encased in solid ice at non-injurious temperatures during winter dormancy. The more severe injury sustained by ladino appeared to be associated with the larger stolons and the higher level of metabolic activity during winter dormancy.

The overwintering of such legumes as alfalfa, sweetclover, and red clover has been shown to be associated with a high level of carbohydrate storage and with subsequent changes in certain carbohydrate components (2, 3, 7, 8, 9, 10, 16, 17, 19). Wood and Sprague (21) at New Jersey have reported that the more cold-hardy clones of ladino clover were higher in most carbohydrate fractions during the winter than the less hardy ones. Changes in the levels of sucrose, total sugars, and total polysaccharides in the hardy clones were made at a more gradual rate during winter. Fall cutting decreased some carbohydrate fractions and increased others but had no measurable influence on low temperature survival. Tesar and Ahlgren (18) at Wisconsin have shown that the percentages of total available carbohydrates in ladino clover stolons in November were not significantly different even though various heights and frequencies of cutting were applied during the growing season.

The investigations reported here deal with the trends of cold resistance and the trends of certain carbohydrate fractions from fall to spring in the overwintering parts of medium red, ladino, and common white clover, and the relationship of these trends to the prevailing weather. The studies were made at Madison, Wisconsin, during the winters of 1952–53 and 1953–54.

MATERIALS AND METHODS

Wisconsin-grown seed of medium red clover, *Trifolium pratense* L., and of ladino and common white clover, *Trifolium repens* L., was seeded in rows and in broadcast plots during June of 1952 and of 1953. Plants grown in rows were used in the cold tests initi-

[1] Received April 6, 1956.
[2] Published with approval of the Director, Wisconsin Agricultural Experiment Station.
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ated in early September. Ladino and white clover plants were sampled directly from the rows throughout the fall, winter, and spring. During the winter when the soil was frozen, red clover plants were used which had been dug in late November, placed in pots of soil, and buried in the field. The crowns were left exposed to about the same degree as if the plants had remained undisturbed in the field.

The crown and a portion of the root of red clover and the stolons of ladino and white clover were used in the cold tests. The red clover roots were trimmed to 5 cm below the initial crown branches and the top growth to about 1.5 cm above this point. Stolons of ladino and white clover were severed 10 cm from the terminal bud, and all leaves and roots were removed.

The electrical conductance method described and used by Dexter et al (4, 5, 6) was used to measure the degree of cold resistance in the tissue. Nine 5-gm samples were prepared for each legume at each sampling date. Each sample was placed in a 25 × 200 mm test tube stoppered lightly. Five samples were frozen for 14 hours at −8° C. The remaining 4 samples were held at 2° C to serve as checks. The samples were then immersed in 50 ml of distilled water for 20 hours at 2° C. Specific conductance readings were made on the liquid from around the tissue after bringing the liquid to 25° C.

The frozen and unfrozen tissues used in the tests were planted in the greenhouse in flats of pit sand. After 21 days, the dry weight of the leaves produced by the frozen tissues was compared with the weight produced by the unfrozen tissues with the latter scored as 100 %.

The seedings made in broadcast plots were subjected to four different management treatments during the fall months. One set of plots was cut four times at two-week intervals beginning September 15; another set was cut three times at two-week intervals beginning October 1; another set was cut on October 15 and on November 1; and another set of plots was not cut during the fall months. The last cutting in each of the cutting treatments was made on November 1.

Samples were taken from the plots at monthly intervals from late November to late March by removing all plants within a random 2-square foot area (0.186 m^2) in each plot at each sampling date. Red clover plants used after the soil became frozen were from samples dug in late November and buried in the plots as described previously.

Samples dug at the first sampling in November and at the sampling in late March were separated into top and root growth. The red clover top growth was trimmed of all leaves and elongated stems so that only the crown and the young tiller branches remained. All of the crown and stolon growth of the ladino and white clover was used after removing the leaves. Roots were cut to a length of 20 cm. Only the stolons of ladino and white clover and the roots of red clover were used from the samples taken in December, January, and February. The plant material was washed, oven-dried, finely ground, and later analyzed for carbohydrates.

Analyses for total available carbohydrates were made by extracting with the Weinmann method (20) using the modifications of Lindahl et al (13). Reducing and total sugars were extracted as outlined by the Assoc. Offic. Agr. Chemists (1). Starch was obtained by subtraction. Hemicellulose was extracted by 10 % hydrochloric acid hydrolysis of the residue remaining after the total available carbohydrates were extracted. The reducing power of the extracted carbohydrates was determined by the Shaffer-Somogyi method outlined by Heinze and Murneek (11). The carbohydrates were calculated as glucose on a dry weight basis.

Results and Discussion

Trends of Cold Resistance: The trends of cold resistance, as measured by electrical conductance, during the two winters of study for each legume were in general quite similar. However, the data during the winter of 1953–54 showed considerable variability within and among samples since the ladino and white clover stolons in particular were severely desiccated as a result of a very dry fall and of little winter snow cover. Therefore, only the data for 1952–53 are presented. The average specific conductance readings from early fall of 1952 to late spring of 1953 are shown in the lower part of figure 1, and the average weekly air temperatures and inches of snow cover are shown in the upper part. Decreasing conductance readings indicate increasing cold resistance and vice versa.

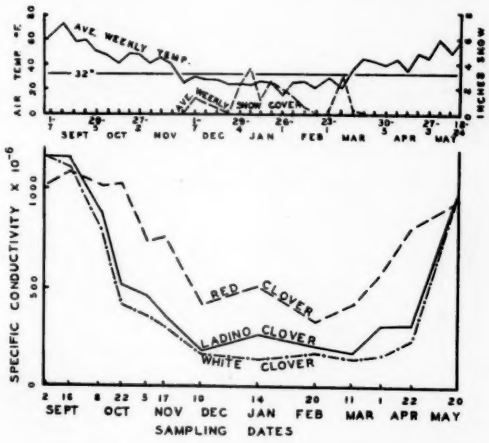

Fig. 1. Specific conductance in reciprocal ohms of the water extracts of field-hardened roots and crowns of medium red clover and the stolons of ladino and white clover from plants grown in rows at Madison, Wisconsin as measured from fall to spring of 1952–53 following 14 hours of freezing in the laboratory at −8° C, and the average weekly air temperatures and inches of snow cover in the field.

The trends of cold resistance, as measured by electrical conductance, of the white and ladino clover were similar with white clover showing more resistance than ladino. Red clover was considerably less cold resistant than ladino or white clover throughout the entire sampling period.

Ladino and white clover developed cold resistance steadily from mid-September to early December when a near maximum for each was reached. The most rapid development occurred between mid-September and late October. Red clover did not develop a measurable degree of resistance until after mid-October. Thereafter, resistance increased until a near maximum was reached at the December 10 sampling as with the ladino and white clover.

With steadily decreasing air temperatures, the soil surface became permanently frozen after November 27. The first substantial snow fall occurred on December 1. Therefore, the near maximum of cold resistance reached in all three species at the December 10 sampling occurred after the soil surface became frozen and after the average weekly air temperatures remained below freezing. The substantial snow cover which prevailed in early December no doubt influenced the maximum level of resistance which was developed in each legume.

A high level of cold resistance, as measured by electrical conductance, was maintained in all three clovers from mid-December to mid-March. Some variability in resistance apparently did occur, however, depending on snow cover as indicated by the greenhouse recovery of the frozen tissues (fig 2). During this period, the plants were protected from the low air temperatures by a substantial snow cover except for a two-week period in late February. The recovery in the greenhouse of the tissues sampled from the field during the period of heavy snow cover showed an increase in injury. Cold resistance then increased again apparently during late February when the snow cover was light or absent.

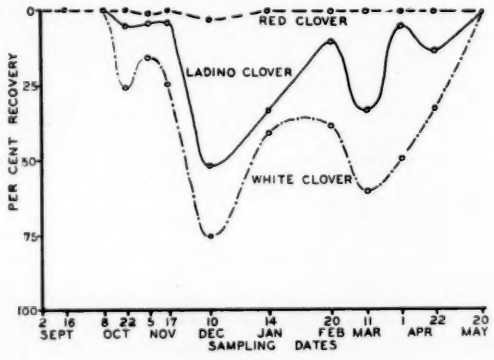

FIG. 2. The percentage recovery of plants frozen at −8° C for 14 hrs based on the weight of leaves produced following 21 days growth in the greenhouse with the growth of unfrozen plants scored as 100 %.

TABLE I

THE AVERAGE AMOUNTS OF TOTAL AVAILABLE CARBOHYDRATES IN THE OVERWINTERING PARTS OF MEDIUM RED, LADINO, AND COMMON WHITE CLOVER IN THE LATE FALL AND EARLY SPRING OF 1952–53 AND 1953–54 OVER ALL CUTTING TREATMENTS

CLOVER SPECIES	PLANT PART	TOTAL AVAILABLE CARBOHYDRATES				% LOSS FROM FALL TO SPRING	
		NOVEMBER		MARCH			
		%	gm*	%	gm*	%	gm*
Red	Roots	34.3	11.5	18.4	5.0	46	57
	Tillers & crowns	17.6	3.0	10.5	1.6	40	47
Ladino	Roots	28.0	6.4	11.7	1.2	58	81
	Stolons & crowns	35.6	25.0	20.5	6.3	42	75
Common white	Roots	25.8	4.2	14.8	1.2	43	71
	Stolons & crowns	35.1	19.1	25.1	7.1	28	63

* Based on gm of plant tissue from a 2 sq ft field area (0.186 m^2).

Cold resistance in all three species began to decrease after mid-March. This coincided with the disappearance of the snow about March 12 and the thawing of the soil surface about March 18. In red clover, there was a steady decrease in resistance from mid-March to the last sampling made on May 20. However, a marked decrease did not occur in ladino and white clover until after the April 22 sampling.

The amount of leaf growth produced by the plants in the greenhouse following freezing (fig 2) verified the differences in cold resistance found in the conductance tests. Recovery from freezing was greatest in the white clover, least in the red clover, while ladino clover was intermediate. There was an apparent discrepancy between the greenhouse and conductance tests particularly since the red clover showed almost complete killing following planting in the greenhouse. This was probably due to cells that were still alive immediately after freezing when the conductance test was made but died during the three weeks of recovery in the greenhouse.

OVERWINTERING CARBOHYDRATE STORAGE: Samples removed from the field in late November indicated that the highest weights of total available carbohydrates per 2-square-foot area (0.186 m^2) and the highest percentages were in the roots of the red clover while in the ladino and white clover, the highest levels were in the crowns and stolons (table I). This was apparent also in the spring samples. The average loss over winter was greater in both percentage and weight in the roots of the legumes than in the above-ground parts, as was noted also by Jeffers (12). Movement of carbohydrates from the roots to crown tissues may occur during winter. Also, a higher level of metabolic activity may occur in the roots over winter than in the above-ground parts since the roots

are insulated to some extent from the lower temperatures prevailing at or near the soil surface.

INFLUENCE OF FALL CUTTING ON CARBOHYDRATE STORAGE: The fall cutting treatments had no clear cut influence on the levels of the various carbohydrate fractions in the principal storage parts (stolons) of ladino and white clover. However, differences occurred in the principal storage part (root) of red clover. The percentages of total available carbohydrates, starch, and total sugars in the red clover roots were in general highest in plants not fall cut and were lowest in plants which had been cut, particularly in those cut four times during the fall period.

The lack of any marked influence of fall cutting on the carbohydrates in ladino and white clover was due in part to the extremely dry conditions which prevailed during the fall of both years. Ladino and white clover made little or no growth following cutting. Red clover with a deeper root system was able to obtain moisture from the deeper soil levels, and it made some growth following each cutting. The leaves of red clover were also virtually all removed with each cutting, but many leaves near the soil surface on ladino and white clover were not removed because of the prostrate growth of the stems. As a result, the cutting treatments were more effective in removing photosynthetic tissue and in depleting carbohydrates in red clover than in ladino and white clover.

CARBOHYDRATE TRENDS: Even though fall cutting failed to exert any clear cut influence on the various carbohydrate fractions, the data show the trends of these fractions during the winter period in the principal storage parts of the legumes. The percentage trends from late fall to early spring of 1952–53 in the roots of red clover and in the stolons and crowns of ladino and white clover are shown in figure 3. Only the data for the one year are presented since the trends were similar during the two years of study.

Throughout all samplings in each clover, the percentages of reducing sugars were very low (less than 2.5 %). There was also little fluctuation in the percentages of reducing sugars or of hemicellulose from late fall to early spring.

The percentages of total available carbohydrates in the stolons and crowns of white clover declined steadily over winter as they were used in respiration. Starch was at a high level at the time of the first sampling on November 24, and it declined only slightly to late December. Thereafter, starch declined rapidly as it was converted to sugars. Total sugars increased steadily from the initial sampling and reached a maximum in late February. After late February, the total sugars declined also.

As in white clover, the percentage of total available carbohydrates in the stolons of ladino clover declined steadily over winter. The high level of starch found at the initial sampling declined rapidly during December as it was converted to sugars, and thereafter, the decline occurred more slowly. Total sugars reached a maximum in late December or early January after which they declined. Thus, the conversion of starch to sugars and the maximum level of total sugars occurred earlier in the ladino clover stolons than in the white clover stolons.

The percentage of total available carbohydrates in the roots of red clover declined steadily over winter as with the ladino and white clover. Starch was already at a lower level with the initial sampling on November 24 than in white or ladino clover, but there was a steady decline over winter. However, the per-

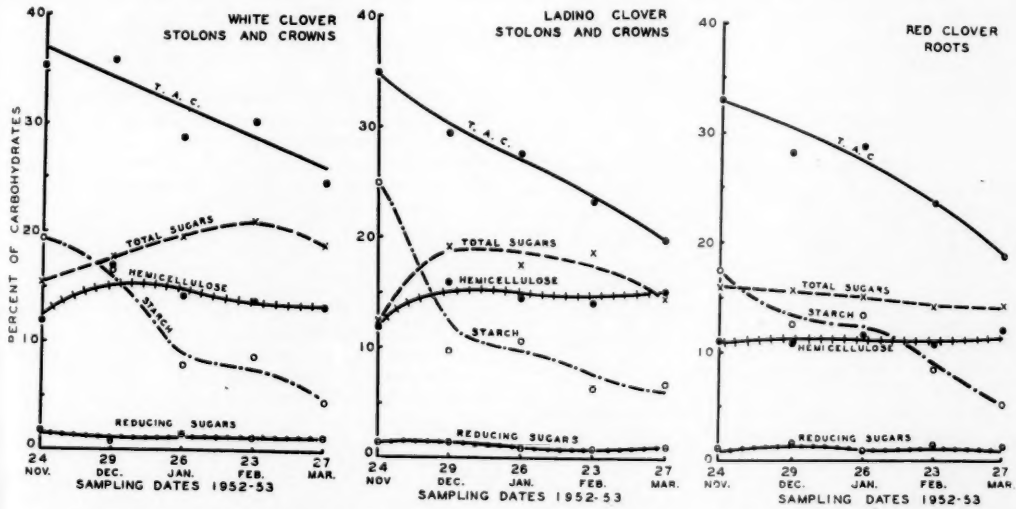

FIG. 3. The percentages of total available carbohydrates, reducing sugars, total sugars, starch, and hemicellulose in the stolons and crowns of common white and ladino clover and in the roots of medium red clover from fall to spring of 1952–53.

centage of total sugars declined less than 2% from fall to spring. The major conversion of starch to sugars and the maximum level of total sugars were apparently reached prior to the first sampling in late November.

The data for two winters indicated that the starch to sugars conversion occurred earlier in the fall and winter in red clover than in ladino or white clover. Red clover was found also to be the least cold resistant. The highest level of total sugars was found in white clover with the maximum occurring in late winter, and white clover was the most cold resistant. The level of total sugars in ladino clover was intermediate between red and white clover, and its level of cold resistance was also intermediate. It appeared that the conversion of starch to sugars occurred later and the total sugars were at a higher level during winter dormancy in the more cold resistant species.

Summary

Cold resistance in common white clover stolons developed more rapidly and reached a higher level than in ladino clover stolons although the trends were similar. Cold resistance developed later and more slowly and did not reach as high a level in the roots and crowns of medium red clover.

Most of the cold resistance was developed in each clover soon after permanent soil freezing and after the average weekly air temperatures began to remain below freezing. A high level of resistance was maintained from mid-December to mid-March with some variability depending on the amount of snow cover. Cold resistance began to decrease after mid-March by which time the snow cover was gone and the soil surface was thawed. A marked decrease did not occur in ladino and white clover until late April.

The principal parts for the storage of available carbohydrates were the primary root in red clover and the stolons and crown in ladino and white clover. Loss of carbohydrates over winter was greater in the roots than in the above-ground parts.

Very little fluctuation occurred over winter in the percentages of reducing sugars or hemicellulose in the principal storage parts. Total available carbohydrates declined steadily over winter accompanied by a conversion of starch to sugars during the late fall or winter. Conversion of starch to sugars occurred later and the total sugars were at a higher level during winter dormancy in the more cold resistant species.

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FLUORESCENCE–SPECTRUM CURVES OF CHLOROPHYLLS, PHEOPHYTINS, PHYCOERYTHRINS, PHYCOCYANINS AND HYPERICIN[1]

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Any quantitative investigations of fluorescence phenomena more or less presuppose some information as to the spectral emission curve of the substances under investigation. Knowledge of the shape of the spectral emission curves of pure substances is necessary to various degrees of accuracy for the identification of pigments by fluorescence, for the detection of fluorescent impurities in mixtures, for the appropriate selection of filters or photocells to measure the fluorescence intensity, and for planning experiments so as to avoid reabsorption of the fluorescence within the sample. Reliable curves for the pure substances are particularly necessary for the analysis of the fluorescence spectra of complex fluorescent mixtures or of living cells. The fluorescent properties of chlorophyll have been investigated from many points of view since the fluorescence of chlorophyll was discovered by Brewster in 1834. Stokes, who discovered the basic principles of fluorescence, also found that chlorophyll fluoresces in leaves. In spite of the widespread occurrence in nature of chlorophyll and its scientific importance we know of only one investigation, Zscheile and Harris (32), reporting the precisely measured fluorescence spectra of pure chlorophylls a and b. The fluorescence spectrum of bacteriochlorophyll in solution has been measured by Vermeulen, Wassink, and Reman (27) and in the intact bacteria by Duysens (9). The present paper revises slightly the calculations upon which Zscheile and Harris based their curves and presents precise curves for other plant pigments, particularly those concerned in the process of photosynthesis.

The yield of chlorophyll fluorescence in polar solvents has recently been found by Livingston and Forster (18) to be 25 %, in contrast to the older accepted value of 10 %. The low fluorescence in pure non-polar solvents and the enhancement of its intensity by traces of polar solvents has been studied by Livingston, Watson, and McArdle (19). Various aspects of chlorophyll fluorescence have been reviewed by Rabinowitch (22), De Ment (5), Pringsheim (21), and Förster (10). Fluorescence spectroscopy provides a very sensitive method for the identification and quantitative determination of the various chlorophylls and other fluorescent substances in plant extracts (13, 14, 15, 23, 29, 30).

Fluorescence spectroscopy of photosynthetic pigments in live plants has been used in studying energy transfer from one pigment to another by Dutton, Manning and Duggar (7), Wassink and Kersten (31), van Norman, French and Macdowall (26), French and Young (12), and Duysens (8, 9). The interpretation of the fluorescence spectra of live plants containing several fluorescent pigments and the use of these spectra in the study of energy transfer depend upon a knowledge of the fluorescence spectra of the individual pigments. Most of the previous investigators of the spectral characteristics of the photosynthetic pigments used visual or photographic observation of the wavelength position of the peaks. Much valuable exploratory work of this type was done by Dhéré and his collaborators (6). Visual and photographic methods are reasonably adequate for measuring the wavelength of major emission bands but are poor for the lesser maxima and often entirely inadequate for those bands that show only as a shoulder on a more intense band. Careful photometry of photographic fluorescence spectra, even when combined with the best calibration procedures, is less precise than the direct photoelectric spectrophotometric measurement of fluorescence curves except when the fluorescence is so weak that only prolonged photographic exposures can detect it. Furthermore, photographic and visual procedures may show distorted positions of the wavelength of the peaks due to the characteristics of the photographic emulsion or of the human eye.

Considering the utility and significance of precise fluorescence curves of biologically important pigments, it is surprising that so few of them have been measured. The chance to make such measurements arose through fortunate coincidences: the pure chlorophylls were on hand through the work of Smith and Benitez (23), the pure phycobilins were prepared for another investigation by Blinks and Airth at the Hopkins Marine Station, and the equipment was on hand from previous work (11, 12, 15, 28, 29, 30).

APPARATUS AND PROCEDURE

The equipment used in these measurements was that of French and Young, modified by Virgin. It has not yet been described in detail, although some of the components are here discussed and previously a block diagram of the apparatus was presented (11). In brief, the apparatus works as follows: The exciting light comes from a high-pressure mercury lamp used with a large grating monochromator or, alternatively, from a medium-pressure mercury lamp with filters. Provision is made for measuring by a thermopile the intensity with which the sample is irradiated. The sample is placed in a holder illuminated on its face at an angle and some of the fluorescent light emitted from the illuminated face is collected by a spherical mirror and passed through another large-grating monochromator to an RCA 6217 photomultiplier. Both monochromators are ordinarily used with a total spectral slit width of 10 mμ. The light from

[1] Received May 1, 1956.

the mercury lamp run on the 60-cycle power line coming in 120-cycle pulses produces voltage pulses of this frequency from the photomultiplier. After AC amplification this voltage is attenuated by a potentiometer driven by a cam linked to the monochromator's wavelength drive. This cam introduces corrections for change in sensitivity of the photomultiplier with wavelength, etc. The partially corrected signal then goes to a photoelectric curve follower which compensates for the small though highly reproducible residual errors in the cam. The 120-cycle signal is selected from random noise by a tuned amplifier, rectified, and recorded on a Brown millivolt recorder whose strip chart paper is driven by the monochromator's wavelength drive motor through selsyn coupling. The linear wavelength scale on the recording paper is such that the 1/3″ lines represent 5 mμ. The cam is shaped and the curve on the photoelectric follower drawn so that the apparatus plots correctly the energy distribution for the light from a standard tungsten lamp reflected from magnesium oxide. Filters are used to remove from the fluorescence emission stray light of wavelengths shorter than 570 mμ. The usable range extends from 570 to 770 mμ with this photomultiplier tube. This range was chosen as being suitable for the fluorescence of photosynthetic plant pigments and allows the use of incident light from 560 mμ to shorter wavelengths. Fluorescence excitation spectra may be measured with this equipment from 400 to 560 mμ. Without the correcting mechanism and the stray light filters the equipment has been used for measuring uncorrected fluorescence spectra from about 430 to 770 mμ. In general the intensity measurements appear to be correct within a few percent. We have not attempted to make absolute measurements of fluorescence intensity or yield.

Several different types of sample cells were used, for the most part a 1-cm rectangular Beckman cell which had on its face a slit 1 mm wide between two parallel strips of aluminum foil cemented to the glass. The slit served to restrict most of the fluorescent light effectively emitted from the cell to a layer 1 or 2 mm in depth. This effect is achieved because the fluorescent light is collected only from the volume of the solution in which the incident and emergent beams cross each other. This active volume is restricted to that near the face of the cell by the slit in the aluminum foil. Thus most of the emitted light has to pass through only a thin layer of solution which avoids the reabsorption of the fluorescent light by the solution. Another arrangement used for smaller samples was a Lucite block milled to a depth of 0.5 mm and covered by a microscope slide. With this cell a covering slit was unnecessary due to the thinness of the layer. Cells of adjustable thickness similar to those used in infrared work were also tried but were found to be less convenient. All of the measurements were carried out in redistilled solvents which were neither dried nor deoxygenated.

It is possible to correct for the distortion introduced by the reabsorption of fluorescent light within the solution by equations given by Förster (10) and Duysens (9). In practice, however, we find it more convenient to make measurements with successive dilutions in approximate steps of two with a given solution until two curves of identical shape are obtained. In this way one is sure of avoiding reabsorption distortion and the uncertainties involved in the calculation are avoided. The curves here reported are considered to be free of reabsorption distortion. The linearity of the recording system was checked by reducing the deflection with calibrated screens at the peak or at other significant wavelengths of the fluorescence curve.

The absence of stray light, particularly of the exciting wavelength, in the fluorescence spectra was demonstrated by substituting magnesium oxide for the sample and recording the output of the instrument without changing the conditions of measurement such as incident light intensity and amplification. Adequate freedom from stray light was obtained under the conditions used for recording all the curves reported here because of their relatively intense fluorescence. With plant materials containing very small amounts of pigments, this stray light must occasionally be measured and subtracted. The incident wavelength was usually 436 or 405 mμ. With pigments that absorbed more strongly elsewhere other exciting wavelengths were used, e.g., for bacteriopheophytin 525 mμ and for hypericin and some phycobilins 546 mμ.

Results and Discussion

Chlorophylls and Pheophytins: The fluorescence of chlorophylls a and b in ether has been measured with high precision by Zscheile and Harris (31). Dr. L. N. M. Duysens has kindly called our attention to a minor error in the calibration upon which Zscheile and Harris' fluorescence curves are based. The relative response of their photocell to different wavelengths was measured by comparing the photocell response to the thermopile response when both were placed alternately behind the slit of the monochromator. This procedure calibrates correctly the photocell response to different wavelengths. But there are two factors which prevent this type of calibration from giving a precise measure of the energy distribution of the fluorescent light. In the first place, the slit width in terms of millimicrons isolated is not constant throughout the spectrum. Thus at longer wavelengths a larger wavelength region is admitted to the photocell. In regard to the curves of Zscheile and Harris, figure 4, it is stated that the monochromator slits were 0.2 mm wide. This slit width isolated spectral regions of 9.2, 10.2, 12.0, and 13.8 mμ at 630, 660, 660, 710 and 760 mμ respectively. Since the amount of energy transmitted is proportional to the band width isolated by the monochromator, these figures have been used to apply the necessary correction for the variable band width. This correction reduces the height of the 730 fluorescence peak of chlorophyll a to the 664 mμ peak by the factor 103.5/123.5 = 0.833. A second possible source of trouble lies in the varia-

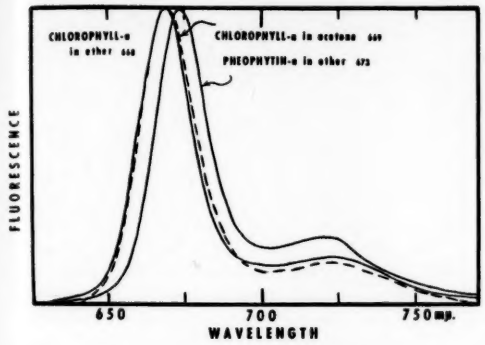

FIG. 1. The fluorescence spectra of chlorophyll a and pheophytin a.

tion of transmission of the monochromator with wavelength, due largely to the variable reflection loss for different wavelengths. Since the total wavelength range covered is small and the necessary data unavailable, we have neglected this minor correction. The curves by Zscheile and Harris recomputed in this way have been published elsewhere in comparison with our chlorophyll a and b curves (23).

The fluorescence spectra of the chlorophylls are shown in figures 1 to 5. These measurements were made with the highly purified chlorophylls prepared by Smith and Benitez (23, 24). Most of the measurements were made in ether, although a few curves in acetone are included. The fluorescence maximum for chlorophyll a dissolved in ether was found by us to be 668 mμ, by Livingston, Watson and McArdle (19) 666 mμ, and by Zscheile and Harris (32) 664.5 mμ. The difference in the positions of the fluorescence peaks found by us and by Zscheile and Harris is consistent with the difference in the absorption peaks also observed by the two groups. The discrepancies while small are outside the experimental errors of the wavelength measurement. They probably indicated differences in the isomeric composition of the chlorophyll or in complex formation with the solvents.

The fluorescence spectrum of pheophytin c in ether shown in figure 3 is quite different from that of pheo-

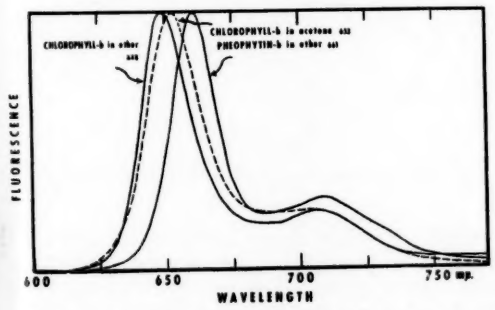

FIG. 2. The fluorescence spectra of chlorophyll b and pheophytin b.

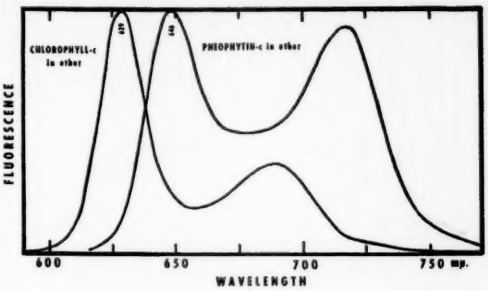

FIG. 3. The fluorescence spectra of chlorophyll c and pheophytin c.

phytins a, b, and d, whose fluorescence spectra more closely resemble those of the corresponding chlorophylls. The strange shape of this curve may be due to the possibility of some effect of acid upon chlorophyll c other than the mere removal of magnesium. It is, however, similar in shape to the spectrum of protopheophytin shown in figure 5. This protopheophytin curve may be less reliable than the others in this paper because the material was an unpurified acid-acetone extract of the inner seed coats of squash. Acetone extracts of similar seeds, however, showed light absorption in the orange and red part of the spectrum to be due only to protochlorophyll, so it is highly likely that no other orange or red fluorescing substances other than protopheophytin were present in this extract.

In figure 6 is given the fluorescence of purified bacteriopheophytin in ether and the spectrum of a green pigment found in partially purified preparations of bacteriochlorophyll. The separation of this green pigment has been described by Smith and Benitez (23) and by Holt and Jacobs (17). This green pigment is responsible for the peak at about 700 mμ in the fluorescence curve (measured with wide slits) for bacteriochlorophyll reported by Vermeulen et al (27).

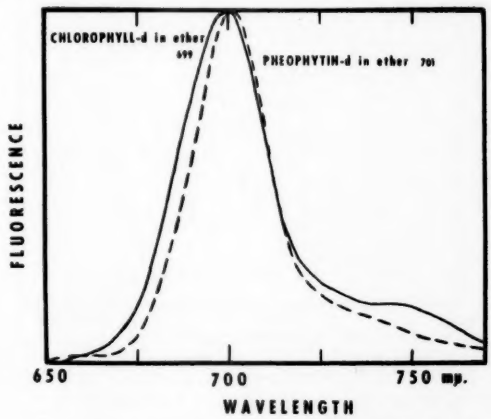

FIG. 4. The fluorescence spectra of chlorophyll d and pheophytin d.

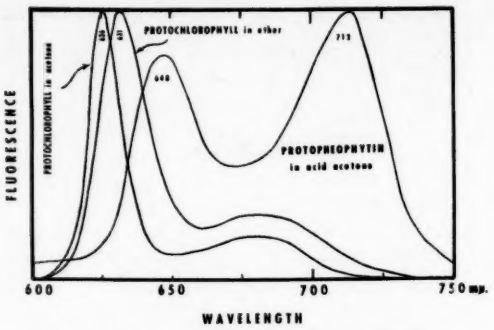

FIG. 5. The fluorescence spectra of protochlorophyll from barley and of protopheophytin in an acid acetone extract of a squash seed coat. The latter curve was made in collaboration with Dr. Robert Hill.

Purified preparations of bacteriochlorophyll do not show this fluorescence peak at 687 mμ. Since the main peak of bacteriochlorophyll comes at a longer wavelength than 770 mμ, we were not able to measure it.

PHYCOBILINS: All phycobilin pigments were isolated from *Porphyra naiadum*. They were separated by column chromatography using the method of Swingle and Tiselius (25) with the omission of sucrose, which is necessary only to carry out a frontal analysis. In addition one sample of phycoerythrin was obtained by crystallization. The absorption spectra of phycocyanin and allophycocyanin, each of which had been rechromatographed once, and of crystalline phycoerythrin are shown in figure 7. The preparation and properties of these pigments have been described by Airth (1). It is apparent from these spectra that the phycocyanin was slightly contaminated by phycoerythrin and that the allophycocyanin contained considerable phycocyanin. About 10 % of the total light absorbed by the allophycocyanin solution at 546 mμ was due to phycocyanin. Blinks has tentatively designated the phycoerythrin from *P. naiadum* as B-phycoerythrin. The allophycocyanin from several species of red algae, which was isolated and described by Haxo, O'h Eocha and Norris (16) had absorption maxima at 650 mμ; whereas the allophycocyanin described here has its maximum absorption at 654 mμ (pH 5 to 7). The phycocyanin from *Porphyra naiadum* has the absorption characteristics of Cyanophyta phycocyanin even though this alga belongs to the division Rhodophyta.

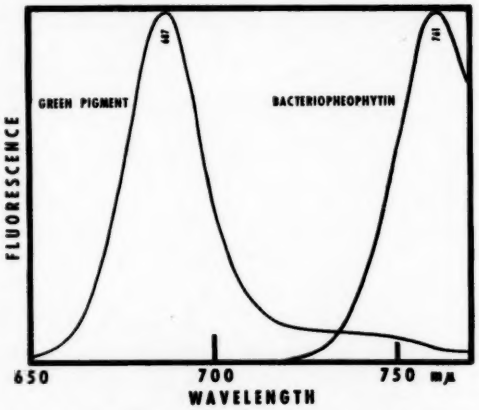

FIG. 6. The fluorescence spectra of bacteriopheophytin and of the green pigment found in preparations of bacteriochlorophyll before final purification. This green pigment is probably acetyl chlorophyll a, which is also called bacterioviridin.

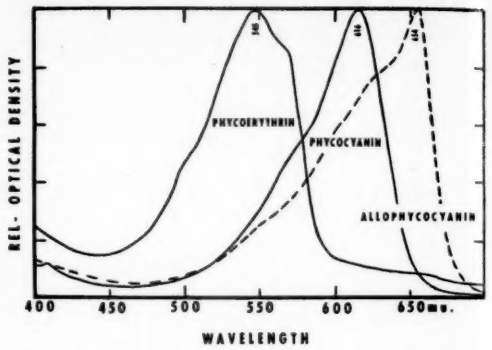

FIG. 7. The relative absorption spectra of the phycobilin pigments isolated by Airth (1) from *Porphyra naiadum*. The phycoerythrin was obtained by crystallization and the others by chromatography. These solutions were used for the fluorescence spectra of figure 8.

After determining the absorption spectra, the same phycobilin solutions were used for the fluorescence spectrum measurements presented in figure 8. For the chromatographically isolated compounds the pH was 5.0; in the case of crystalline phycoerythrin the pH was 7.0. The absorption spectra of the two phycoerythrins were similar except that the minor band at about 565 mμ was higher in the chromatographically isolated phycoerythrin. The height ratios

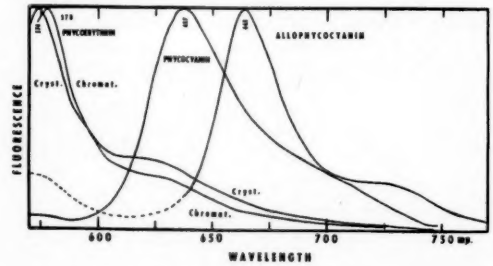

FIG. 8. The fluorescence spectra of the phycobilins from *Porphyra naiadum*. A phycoerythrin preparation purified by crystallization gave a fluorescence peak at 574 mμ while that prepared by chromatography had its peak at 578 mμ like most of the other phycoerythrins that we have measured.

at 565 and 545 mμ of the crystalline phycoerythrin was 0.87 while that for the chromatographic phycoerythrin was 0.93. Furthermore, chromatographic phycoerythrin always contained a phycocyanin impurity which was impossible to remove. In this sample of phycoerythrin the phycocyanin impurity absorbed about 1 % as much at 546 mμ as did the phycoerythrin itself. The fluorescence maximum at 637 mμ for phycocyanin is considerably less than that deduced from the spectrum of the intact algae by French and Young (12) in Porphyridium.

Duysens (9, fig. 6.2) has given the fluorescence and absorption spectra of Oscillatoria phycocyanin. Both absorption and fluorescence spectra for the partially purified phycoerythrin from Porphyridium have been published (11). Corresponding curves for the pure C-phycoerythrin from Porphyra, prepared by Blinks, are also included in (11). We know of no other fluorescence spectrum of allophycocyanin. Absorption curves of phycoerythrin from different sources may show one, two or three peaks; the fluorescence spectra of all phycoerythrins are, however, nearly identical.

Hypericin: The absorption spectrum, and photodynamic action of hypericin which sensitizes animals to light has been given by Blum (3). Its fluorescence has been qualitatively investigated by Dhéré (6). Its synthesis has been achieved by Brockman and Muxfeldt (4), and its absorption spectrum measured by Pace and Mackinney (20).

The yellow flowers of *Hypericum perforatum* in the intact state show no fluorescence. When they are immersed in methanol a non-fluorescent yellow pigment comes out. If this solution is allowed to stand for a day or so it darkens and the characteristic fluorescence of hypericin appears. The preparation used was obtained by crushing about twenty flowers with a glass rod and allowing the brei to stand, after which it was extracted with 95 % ethanol. After about two hours the clear red extract was washed several times with small portions of petroleum ether in a separatory funnel to remove the chlorophyll pigments, a small amount of water being added to separate the two phases. The red pigment solution was then evaporated to about 10 ml and an equal volume of absolute alcohol was added. This gave a crude preparation containing hypericin and perhaps also pseudohypericin. Its fluorescence curve is given in figure 9.

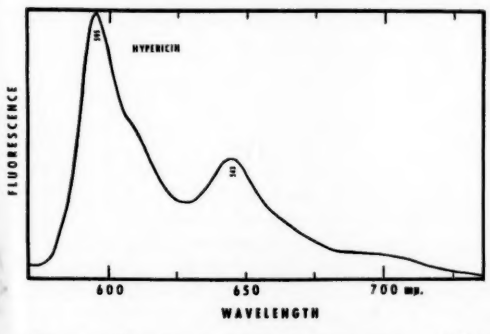

Fig. 9. The fluorescence spectrum of a partially purified ethanol extract of the petals of *Hypericum perforatum*.

Summary

The spectral curves of fluorescence emissions for the following pigments have been measured and are presented in this paper: chlorophylls a, b, c, d, and protochloropyll and their corresponding pheophytins; bacteriopheophytin; phycoerythrin, phycocyanin, allophycocyanin; and hypericin.

Numerous discussions with Professor Lawrence R. Blinks and the use of the phycobilin preparations from his laboratory which made one phase of this work possible are gratefully acknowledged. Dr. L. N. M. Duysens was a member of the laboratory group during most of the time that these measurements were being made and we are indebted to him for many helpful comments. Earlier fluorescence curves for many of the substances reported here were measured by Dr. Violet Koski Young, who also collaborated in the development of the procedures. Improvements in the equipment after she left as well as the greater purity of some of the compounds later available, however, led us to base this paper mainly on the more recent data. The electronic components were designed, revised, and tested by Messrs. B. G. Ryland, H. C. Patton, Jr., D. R. Schuech and G. H. Towner. The mechanical parts of the monochromator were constructed by Mr. Frank Schuster. We wish to thank all of these men for the continued reliability of the apparatus over a six-year period.

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UTILIZATION OF BETAINE AS A METHYL GROUP DONOR IN TOBACCO[1,2]

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The methyl groups of choline were shown to serve as precursors for the N-methyl group of nicotine in three-month-old photosynthesizing tobacco plants (3). On the other hand, choline yielded no methyl groups to hordenine in barley seedlings (9), to ricinine in etiolated castor bean seedlings (7) or to protopine in young Dicentra hybrids (11). It was suggested from these studies that the success or failure of choline to be a methyl group precursor in different species might be related to the presence or absence of a choline oxidase which would catalyze the oxidation of choline to betaine. If the oxidation of choline to betaine were first necessary for methylation to occur, then betaine might act as a methyl group donor in plants which could not use choline. Sribney and Kirkwood (12) recently tested this possibility and found that in barley seedlings betaine methyl groups served as precursors for the methyl groups of N-methyl tyramine and hordenine. However, in sprouting castor beans, betaine failed to supply methyl groups to ricinine (12). The question, therefore, as to whether choline is first oxidized to betaine before it can serve as a methyl donor in higher plants is not completely answered.

[1] Received May 3, 1956.

[2] The data in this paper were taken from a thesis presented by C. S. Sato as partial fulfillment of the requirements for the Ph.D. degree.

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It was of interest in the present study to investigate the role of betaine as a methyl donor in photosynthesizing tobacco plants and to attempt to see whether or not choline might be oxidized to betaine before transmethylation occurred. It was shown that the carbon of betaine methyl groups gave rise to the methyl group of nicotine in tobacco. Betaine methyl groups were incorporated into nicotine to about the same extent as choline methyl groups. After radioactive choline was fed to tobacco plants, it was possible to isolate radioactive betaine and dimethylglycine. This finding indicated that in tobacco plants some choline is first oxidized to betaine before methylation occurs.

Methods, Materials and Results

The betaine-methyl-C^{14} chloride used in the feeding experiments was synthesized by the method of Ferger and du Vigneaud (8).

Anal. Calc. for $C_5H_{12}O_2NCl$: Cl, 23.08. Found: 23.20.

The radioactive betaine was fed to tobacco plants (*Nicotiana rustica* var. *humilis*) in a nutrient solution as described previously (2).

Uptake of Betaine from a Nutrient Solution: Before feeding C^{14}-labeled betaine it was necessary to ascertain the extent of uptake of betaine through the roots and its possible destruction by microorganisms on the plant roots. Twelve 125-ml Erlenmeyer flasks were prepared each containing 25 ml of nutrient medium (3), 0.5 ml of a solution containing 0.5 mg of aureomycin and 2.06 mg of betaine chloride. The roots of intact plants about 6 cm high and with a well developed root system were placed in 4 of the flasks, about 6 root fragments 1 cm in length were placed in another 4 and the remaining 4 flasks served as controls. Seventy-two hours later the plants and root fragments were removed and washed, the washings added to their respective flasks and the solution filtered. The filtrates were then analyzed for remaining betaine by the method of Reifer (10). It was found that 98.5 % of the betaine originally present in the solution containing the roots of intact plants had disappeared in the 72-hour period. The disappearance of betaine in the flasks inoculated with root fragments and in the controls was negligible. These facts were interpreted to mean that no destruction of betaine by root microorganisms occurred in the nutrient solution and that betaine was readily absorbed through the roots.

Administration of Betaine-Methyl-C^{14}: Betaine-methyl-C^{14} chloride was then fed to two groups of tobacco plants. Each plant received 2.06 mg having a count of 1.92×10^5 cpm as measured with an internal gas flow Geiger-Müller tube which had an efficiency of 20 %. After a 7-day feeding period the plants were removed from the nutrient solution and nicotine was isolated as the dipicrate by a procedure described previously (2). The radioactivity of the nicotine dipicrate obtained in the two experiments is presented in table I. The radioactivities are expressed as cpm/millimole at "infinite thinness." It may be seen that the nicotine was radioactive after feeding betaine-methyl-C^{14}.

Table I

Radioactivity of Nicotine after Administration of Betaine-Methyl-C^{14}

Expt no.	Maximum specific activity	
	Nicotine dipicrate	Methyltriethylammonium iodide
	cpm/millimole	
1	1.97×10^3	2.05×10^3
2	1.61×10^3	1.78×10^3

Location of Radioactivity in the Nicotine Molecule: Since the nicotine isolated from the betaine-methyl-C^{14} fed plants was radioactive, it was of interest to ascertain the amount of the total radioactivity located in the N-methyl group. The nicotine was, therefore, cleaved according to the procedure of Brown and Byerrum (2), and the methyl group was recovered as methyltriethylammonium iodide. The radioactivity of this quaternary salt obtained from the nicotine in the two experiments is also presented in table I. It is apparent that within experimental error all of the radioactivity of nicotine following feeding of methyl labeled betaine is in the N-methyl group.

Administration of Choline-Methyl-C^{14}: In an attempt to ascertain whether or not choline might first be oxidized to betaine before it could yield its methyl groups for nicotine synthesis, it was decided to feed radioactive choline to tobacco plants and after a suitable period to try to isolate radioactive betaine from the plants. Choline-methyl-C^{14} chloride was synthesized by the method of Christenson and Tolbert (4), and was recrystallized immediately before feeding.

Each of 14 tobacco plants about 3 months old was first prepared by cutting off the roots and allowing the regeneration of new roots in the synthetic nutrient medium for a period of one week prior to feeding the radioactive choline. This procedure was used because it had been found that the newly growing root tips rapidly synthesized nicotine and, therefore, presumably would rapidly metabolize other compounds. Each plant was then administered, in the nutrient medium, 2.0 mg of choline chloride having a radioactivity of 10^5 cpm. One week later the plants were removed from the flasks in which they had been growing, cut into small pieces, and dropped into 500 ml of boiling 0.02 M HCl to inactivate the enzymes. After the mixture had cooled, it was homogenized in a Waring Blendor for 15 minutes and then filtered through an extraction thimble. The thimble containing the residue was transferred to a Soxhlet apparatus and was extracted for 7.5 hours using the acidic filtrate, obtained from the filtration, as the extraction solvent. The clear extract was then treated with 25 ml of 1 *N* NaOH and the solution thus obtained was concen-

trated to about 200 ml in vacuo at 50° C. Nicotine was removed during this concentration as an azeotropic mixture with water. In order to remove some contaminating substances such as chlorophyll and leaf pigments the cooled, concentrated extract was extracted several times with petroleum ether. The aqueous layer was separated from the petroleum ether, filtered and concentrated to about 25 ml. The solution was then acidified with 6 *N* HCl and an equal volume of freshly prepared saturated aqueous ammonium reineckate added. This mixture was placed in an ice bath for 30 min during which time the reineckates of the various bases precipitated. These crystals were filtered, then dissolved in acetone and the reineckate ion precipitated as silver reineckate by addition of excess silver nitrate solution. After removing the silver reineckate by centrifugation, the clear colorless supernatant was decanted and acidified with hydrochloric acid. The silver chloride precipitate was then removed by centrifugation, the supernatant was decanted and evaporated to dryness in vacuo. Since the residue had a greenish color, it was dissolved in 1 ml of water, and precipitated again with ammonium reineckate. The recovery of the chlorides of the bases from the reineckate was then repeated as described above. A white crystalline residue was obtained in this way.

About 0.125 ml of H_2O was added to the crystalline residue and the solution applied to one corner of a sheet of Whatman No. 1 filter paper. The mixture of chlorides of the bases was then separated using two dimensional ascending chromatography. The first solvent system (solvent I) used was n-propanol : 1 *N* acetic acid (3 : 1) (5); and the second (solvent II), n-butanol : ethanol : acetic acid : H_2O (9 : 1 : 1 : 2). A radioautograph of the developed chromatogram was then made. Three spots appeared on the developed x-ray film. By far the most opaque was a spot which had R_f values of 0.46 in solvent I and 0.32 in solvent II (choline: R_f solvent I, 0.46; R_f solvent II, 0.32). Two spots of approximately equal opacity were also present. One had an R_f value in solvent I of 0.41 and in solvent II of 0.23 (betaine: R_f solvent I, 0.40; R_f solvent II, 0.23). The other spot had an R_f value in solvent I of 0.37 and in solvent II of 0.19 (dimethylglycine: R_f solvent I, 0.39; R_f solvent II, 0.19). These data would seem to indicate that after feeding choline-methyl-C^{14} to tobacco plants radioactive betaine and dimethylglycine arise.

Discussion

The present study shows that administration of betaine-methyl-C^{14} to tobacco plants resulted in the formation of nicotine which possessed radioactivity only in the N-methyl group. Therefore the carbon of the methyl groups of betaine is utilized for the synthesis of the N-methyl group of nicotine. It seems possible that a transmethylation reaction from betaine to nicotine might have occurred but this was not proved. However, it has been shown that the methionine methyl group can be incorporated into nicotine by a transmethylation reaction (6), and it therefore seemed pertinent to compare the extent of incorporation into nicotine of the methyl groups of betaine and the methionine methyl group (2). This can be done if one corrects the radioactivity of the isolated nicotine dipicrate samples for the difference in radioactivity of the tracer dose of betaine and methionine fed to the different groups of plants. In addition, since the betaine used in this work had only one of the three N-methyl groups labeled with C^{14} and since betaine apparently yields only one of its methyl groups in methylation reactions, the probability of yielding the C^{14}-methyl group in a methyl group transfer was one in three. When this probability was taken into consideration, betaine was found to be incorporated to a slightly greater extent than methionine into the N-methyl group of nicotine. Since the methyl groups of methionine and betaine were incorporated into nicotine in about the same quantity, it seemed reasonable to postulate that the methyl group of betaine entered nicotine by a transmethylation reaction. A similar comparison of the extent of incorporation of choline methyl groups and betaine methyl groups into nicotine showed the two precursors to be introduced to about the same extent.

It appeared that the present study along with the work of others might indicate whether or not choline must be oxidized to betaine before it can be a methyl group donor in plants. It had previously been shown that in barley seedlings betaine could serve as a methyl group donor for N-methyl tyramine and hordenine whereas choline could not (9, 12). This finding coupled with the observation of Cromwell and Rennie (5) that seedlings were not able to oxidize choline to betaine suggested that in barley seedlings choline does not supply methyl groups to various alkaloids because it cannot be oxidized to betaine, the methyl donor. However, Kirkwood and coworkers (7, 12) have found that neither choline or betaine can supply methyl groups to ricinine in etiolated castor bean seedlings. On the other hand, the methyl groups of ricinine could arise from methionine in these plants (7). It is possible that a methyl transferase specific for methionine exists in castor bean seedlings but no enzyme specific for betaine is present.

Cromwell and Rennie (5) have also indicated that as some plants mature their ability to convert choline to betaine increases. This finding together with the observation that choline as well as betaine can supply methyl groups for nicotine synthesis in 3-month-old tobacco plants might again suggest that it is necessary to oxidize choline to betaine before methylation can occur (3). The finding of Sribney and Kirkwood (11) that choline did not serve as a methyl group precursor for protopine in growing Dicentra hybrids might indicate that Dicentra is one species which does not develop a choline oxidase in either the seedling or mature state.

In the present work evidence indicates that oxidation of choline to betaine might take place before

methylation can occur. It was found that after feeding choline-methyl-C^{14} to plants, radioactive betaine and dimethylglycine had been produced. Choline could give rise to betaine either by transferring its methyl groups to glycine, or some methylated glycine, to yield betaine, or oxidation of choline to betaine could occur. The experiments of Cromwell and Rennie (5) and of Bregoff and Delwiche (1) seem to indicate that betaine is synthesized in two species of higher plants largely through oxidation of choline and not by methylation of glycine. This finding would suggest that the radioactive betaine found in tobacco plants after feeding radioactive choline resulted from oxidation of choline. If this assumption is correct, the dimethylglycine would arise from betaine which had transferred one of its methyl groups. No radioactive demethylation product of choline was discovered.

An attempt was made to demonstrate choline oxidase activity in various preparations from tobacco leaves and roots but thus far this has been unsuccessful.

Although none of the observations in themselves prove that choline must be oxidized to betaine before methylation can occur, the bulk of experiments indicate that in higher plants choline may yield its methyl groups only upon conversion to betaine, but further work will be needed to settle the point.

Summary

It has been shown that the methyl groups of betaine may be incorporated into the N-methyl group of nicotine in tobacco plant metabolism. The extent of incorporation of betaine methyl groups was about the same as the methyl group of methionine and the methyl groups of choline. When choline-methyl-C^{14} was fed to tobacco plants, radioactive betaine and dimethylglycine appeared. This result was interpreted to mean that a choline oxidase system was present in these plants and that some choline was oxidized to betaine before methylation of nicotine occurred.

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EFFECT OF MICRONUTRIENT DEFICIENCIES ON NICOTINE FORMATION BY TOBACCO IN WATER-CULTURE[1]

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It would appear from a previous report (7) that a large increase in nicotine synthesis took place in tobacco plants when boron supply was deficient. The increased nicotine formation was associated with excessive root branching. A causal relationship seemed probable in view of evidence indicating that nicotine formation is controlled by the root (1, 5). An excellent review of alkaloid synthesis has been recently published by Mothes (4).

[1] Received May 18, 1956.

The results herein presented confirm and extend the previous data to include methods of sampling, season, variety of tobacco and the effect of other micronutrient deficiencies. Some results of micronutrient toxicity on nicotine synthesis are also included. A check was made by means of paper chromatography to determine whether the range and concentrations of alkaloid and of non-alkaloid compounds fluorescing in ultra-violet radiation differed in the control and micronutrient deficient leaves.

Emphasis in interpretation is placed on the data

with green samples since these were subjected to minimum change prior to analysis. Percentage content of alkaloid was revealed as an unreliable guide in the interpretation of responses. Percentage of total alkaloid is stated, however, for each part of the plant together with harvest weight. Reliance in interpretation has been placed on the quantities of total alkaloid found in the plant and its parts.

Experimental Methods

Connecticut Broadleaf (cigar) and Dixie Bright 101 (flue-cured) varieties of tobacco (*Nicotiana Tabacum* L.) were grown in ten-liter jars with nutrient solutions made deficient in micronutrients by calcium carbonate treatment (6). The greenhouse was kept at about 25° C. Micronutrient deficiencies studied included those of Fe, Zn, Cu, Mn, B, and Mo. Samples were usually single plants, selected from each group of triplicates and analyzed at the early flowering stage of the controls. Aside from the controls each plant chosen showed marked and characteristic symptoms of deficiency or toxicity. Samples were assayed green; i.e., immediately on harvest; oven-dried after separation into leaf, stalk, and root at 80° C in a current of air; or air-dried in the greenhouse. In the last method of sampling, the tops and roots were separated before drying. Dry samples were ground to 50-mesh and placed in tightly stoppered bottles. The green samples were extracted with 50 % acetone, using a Waring Blendor. The solution was prepared within 2 hours after harvesting, and without exposure to elevated temperatures. Solutions so prepared can be stored in a refrigerator for a week with no significant change in nicotine and nornicotine content.

The green plant material was subsampled when samples of more than 100 gm were encountered. In the case of leaf samples, all of the leaves of the plant were piled with the midribs parallel and one or more strips ½ to 1 inch wide across the leaf, midway between base and apex, were cut from all the leaves simultaneously with scissors or paper cutter to provide a subsample of 50 to 100 gm. Adjacent or intermediate strips were cut and oven-dried to permit calculation of results on an oven-dry basis. Previous investigation had shown that the composition of strips taken in this manner approach closely the average composition of tobacco leaves, which varies between tip and base and between edge and midrib. The stalk was subsampled by sectioning transversely at intervals of about 1 inch. Sections were selected at regu-

Table I

Total Alkaloid in Green Plants Grown in Micronutrient-deficient Water-Cultures

Deficiency	Plant part	Total alkaloid in green plants							
		Connecticut Broadleaf (spring)				Dixie Bright 101 (fall)			
		Fresh wt	Total alkaloid		T.A./root	Fresh wt	Total alkaloid		T.A./root
		gm	*%*	*mg*	*mg/gm*	*gm*	*%*	*mg*	*mg/gm*
None	Leaf	452.0	0.015	6.8		371.0	0.033	122.4	
	Stalk	263.7	0.006	1.6		402.0	0.007	28.1	
	Root	100.6	0.021	2.1		123.0	0.045	55.4	
	Total	816.2	0.013	10.5	0.10	896.0	0.023	205.9	1.67
Fe	Leaf	27.5	0.023	0.6		....	....	....	
	Stalk	3.2	0.030	0.1		....	....	....	
	Root	3.7	0.018	0.1		2.4	....	....	
	Total	34.4	0.023	0.8	0.22	23.7	0.010	2.4	1.00
Zn	Leaf	96.3	0.031	3.0		116.0	0.025	29.0	
	Stalk	32.5	0.015	0.5		84.9	0.009	7.6	
	Root	45.8	0.007	0.3		49.7	0.012	6.0	
	Total	174.6	0.022	3.8	0.08	250.6	0.017	42.6	0.86
Cu	Leaf	341.5	0.012	4.1		333.0	0.026	86.6	
	Stalk	169.0	0.005	0.8		216.0	0.018	38.9	
	Root	58.5	0.009	0.5		54.7	0.017	9.3	
	Total	569.0	0.009	5.4	0.09	603.7	0.023	134.8	2.46
Mn	Leaf	111.0	0.012	1.3		96.4	0.009	8.7	
	Stalk	14.8	0.015	0.2		20.5	0.007	1.4	
	Root	8.0	0.007	0.1		7.0	0.006	0.4	
	Total	133.8	0.012	1.6	0.20	123.9	0.008	10.5	1.50
B	Leaf	334.5	0.058	19.4		285.0	0.108	310.8	
	Stalk	88.5	0.070	6.2		203.0	0.048	97.4	
	Root	38.0	0.055	2.1		59.9	0.031	18.6	
	Total	461.0	0.060	27.7	0.73	547.9	0.077	426.8	7.13
Mo	Leaf	455.5	0.012	5.5		301.0	0.009	27.1	
	Stalk	249.5	0.005	1.2		273.0	0.003	8.2	
	Root	76.0	0.023	1.7		40.5	0.013	5.3	
	Total	781.0	0.011	8.4	0.11	614.5	0.007	40.6	1.00

TABLE II

TOTAL ALKALOID IN AIR-DRIED PLANTS GROWN IN MICRONUTRIENT-DEFICIENT WATER-CULTURES

DEFICIENCY	PLANT PART	TOTAL ALKALOID IN AIR-DRIED CONNECTICUT BROADLEAF TOBACCO							
		SPRING-GROWN				FALL-GROWN			
		AIR-DRY WT	TOTAL ALKALOID		T.A./ROOT	AIR-DRY WT	TOTAL ALKALOID		T.A./ROOT
		gm	*%*	*mg*	*mg/gm*	*gm*	*%*	*mg*	*mg/gm*
None	Leaf	55.2	0.06	3.3		25.3	0.15	3.8	
	Stalk	55.2	0.03	1.7		32.9	0.05	1.6	
	Root	13.8	0.26	3.6		5.7	0.30	1.7	
	Total	124.2	0.07	8.6	0.62	63.9	0.11	7.1	1.25
Fe	Leaf	39.7	0.09	3.6		17.0	0.23	3.9	
	Stalk	25.2	0.07	1.8		9.5	0.20	1.9	
	Root	8.4	0.26	2.2		3.2	0.15	0.5	
	Total	73.3	0.10	7.6	0.90	29.7	0.21	6.3	1.97
Zn	Leaf	30.3	0.10	3.0		16.4	0.23	3.8	
	Stalk	6.7	0.14	0.9		4.4	0.25	1.1	
	Root	6.2	0.26	1.6		2.9	0.15	0.4	
	Total	43.2	0.13	5.5	0.88	23.7	0.22	5.3	1.83
Cu	Leaf	37.9	0.08	3.0		24.0	0.23	5.5	
	Stalk	23.5	0.06	1.4		16.0	0.08	1.3	
	Root	8.4	0.15	1.3		4.1	0.20	0.8	
	Total	69.8	0.08	5.7	0.68	44.1	0.17	7.6	1.85
Mn	Leaf	3.7	0.06	0.2		6.8	0.11	0.7	
	Stalk	0.6	0.05	0.0		1.4	0.20	0.3	
	Root	0.7	0.10	0.1		0.6	0.20	0.1	
	Total	5.0	0.06	0.3	0.43	8.8	0.13	1.1	1.83
B	Leaf	34.7	0.52	18.0		28.4	0.60	17.0	
	Stalk	3.8	0.91	3.5		5.7	0.60	3.4	
	Root	6.6	0.34	2.2		4.5	0.45	2.0	
	Total	45.1	0.53	23.7	3.59	38.6	0.58	22.4	4.98
Mo	Leaf	33.7	0.06	2.0		21.2	0.11	2.3	
	Stalk	17.1	0.06	1.0		18.2	0.06	1.1	
	Root	5.6	0.14	0.8		4.4	0.25	1.1	
	Total	56.4	0.07	3.8	0.68	43.8	0.10	4.5	1.02

lar intervals to provide a 50- to 100-gm sample, the frequency depending on initial sample size. Adjacent sections were oven-dried to determine moisture. Since the root was smaller, it was sectioned laterally, one-half being analyzed and the other oven-dried to determine moisture.

Each sample was assayed for total alkaloid by a spectrophotometric method (2, method 1) with certain exceptions. In the case of the green samples an aliquot of the acetone solution was added to the still, made acid, and the acetone distilled off before the NaOH and NaCl were added and steam distillation begun. In the tobacco varieties employed more than 90 % of the total alkaloid consisted of nicotine, as determined by paper chromatography (3). Nornicotine was low. The developed papers were also examined under ultraviolet radiation and the location and size of the non-alkaloidal fluorescent spots recorded. These fluorescent spots did not show in daylight and so are not believed to be alkaloidal in nature. Root growth and morphology were followed visually during the course of each experiment. The compositions of the nutrient solutions employed and the intake of each essential element during growth have been published in a previous paper (8).

EXPERIMENTAL RESULTS

The results obtained with green samples assayed immediately on harvest are shown in table I. The Connecticut Broadleaf plants were grown in late spring and the Dixie Bright in late fall. Micronutrient deficiencies decreased yields and led to appearance of characteristic symptoms. Root branching in all deficient cultures except boron was no different than in the controls. Boron deficiency led to the formation of a very large number of secondary roots, which ceased growth after attaining a length of a few millimeters. Other secondaries were then initiated. Boron deficient plants also differed from the controls in that they contained about 2.5-fold the quantity of alkaloid. Both varieties of tobacco responded similarly to micronutrient deficiencies even though grown at different seasons of the year.

The relative quantities and percentages of nicotine in the plant organs were similar in the controls to those in field-grown plants. Most alkaloid was present in the leaf and least in the stalk. This relationship was disturbed with most types of micronutrient deficiency. The change consisted only in an interchange in relative quantities of alkaloid in stalk

TABLE III

PERCENTAGE OF TOTAL ALKALOID IN LEAVES OF CONNECTICUT BROADLEAF TOBACCO GROWN AT DIFFERENT SEASONS AND ANALYZED FRESH AND AFTER OVEN-DRYING

DEFICIENCY	TOTAL ALKALOID IN LEAVES OF CONNECTICUT BROADLEAF TOBACCO			
	SPRING-GROWN			FALL-GROWN
	GREEN FRESH BASIS	80° C OVEN-DRY BASIS *	80° C OVEN-DRY BASIS *	80° C OVEN-DRY BASIS
	%	%	%	%
None	0.011	0.18	0.10	0.10
Fe	0.021	0.28	0.22	0.12
Zn	0.017	0.40	0.23	0.19
Cu	0.009	0.23	0.11	0.18
Mn	0.005	0.20	0.09	0.11
B	0.091	0.50	1.48	0.48
Mo	0.007	0.15	0.16	0.10

* Separate experiments in different years. Note variation in increase of alkaloid in minus boron leaves when boron level and translocation enter as possible factors.

and root. Leaf alkaloid was highest in all cases. Partition of total alkaloid between plant organs—leaf, stalk, root—on a quantity basis may in some cases have been a consequence of differing yields of plant parts.

Total alkaloid/root ratios were computed on the assumption that alkaloid per plant might be related to root weight as well as root branching. The relative values so obtained bring out the same relationships as total alkaloid per plant. Only a deficiency of boron appreciably affected nicotine formation by the plant, according to this method of comparison.

The data of table II are for air-dried plants. The quantities of total alkaloid found in the controls and with each type of micronutrient deficiency showed good agreement between spring and fall-grown plants. Since yields in fall were much lower than in spring, this means that alkaloid was higher in fall on a percentage basis.

Effects of micronutrient deficiencies on total alkaloid with air-dried samples were in good agreement with those found with green samples. Boron deficiency caused a 2- to 3-fold-increase in total alkaloid synthesized per plant. Deficiencies of iron, zinc, copper, manganese, and molybdenum gave total alkaloid yields of approximately the same magnitude. The only exception was the spring-grown manganese deficient plant, which made the least growth of all plants sampled.

Total alkaloid/root ratios had relative values agreeing with those obtained with green samples. The boron deficiency values showed 4- to 5-fold increases over those of the control plants. The ratio values for the other micronutrient deficient plants were of approximately the same magnitude as their respective controls. Total alkaloid/root ratios in spring were only about half those in fall, thus reflecting the lesser growth of root obtained in fall.

Percentages of alkaloid in plant organs were irregular on a relative basis. Any part of the plant might be highest or lowest. On a quantity basis partition between leaf, stalk, and root was much the same as in analyses with green samples.

Similar results as concerns the effects of boron deficiency on nicotine formation were obtained by assays for total alkaloid in green and oven-dried leaves. These results are tabulated in table III. No seasonal influence was found with oven-dried leaf samples. Since moisture in the fresh samples approximated 90 %, alkaloid values comparable to those on the dry basis may be estimated by multiplying by a factor of 10.

One directional paper chromatograms were obtained with green, air-dried, and oven-dried leaf samples during the course of these experiments. The alkaloids identified included nicotine, nornicotine, anabasine, and probably myosmine; i.e., those usually present in field-grown plants. These differed with all chromatograms in range and proportions, as between the controls and each of the micronutrient deficiency samples. Myosmine was usually absent. Nornicotine was present in green leaf samples only when manganese and molybdenum were deficient. It was present in all oven-dried and air-cured samples of leaves.

Other differences between controls and deficient cultures were revealed when the chromatograms were examined with ultra-violet radiation. Characteristic

TABLE IV

EFFECT OF MICRONUTRIENT TOXICITIES ON PRODUCTION OF TOTAL ALKALOID IN DIXIE BRIGHT 101 TOBACCO IN FALL

TOXIC AGENT		GREEN WT		TOTAL ALKALOID PER PLANT		T.A./ROOT
COMPOUND	CONC	TOTAL	ROOT			
	gm/l	*gm*	*gm*	%	*mg*	*mg/gm*
None	....	882.0	122.1	0.0150	132.30	1.08
$FeSO_4 \cdot 7\,H_2O$	0.5	45.3	9.4	0.0411	18.62	1.98
$ZnSO_4 \cdot 7\,H_2O$	0.1	676.0	134.5	0.0441	298.12	2.22
$CuSO_4 \cdot 5\,H_2O$	0.025	70.5	11.2	0.0326	22.98	2.05
$MnSO_4 \cdot H_2O$	0.2	640.0	67.8	0.0143	91.52	1.35
H_3BO_4	0.1	817.0	62.3	0.0166	135.62	2.18
$Na_2MoO_4 \cdot 2\,H_2O$	0.025	790.0	91.0	0.0417	329.43	3.62

differences in size and location of non-alkaloidal fluorescent spots were found to be present in the chromatograms for each of the micronutrient deficiencies.

The method of taking samples influenced the chromatographic spots detectible by ultra-violet examination. These fluorescent spots seemed to be least in the oven-dried and most in the air-dried samples. The air-dried samples had, of course, been permitted the greatest length of time during which autolysis could occur.

The influence of micronutrient excesses on nicotine formation was also determined. The data are listed in table IV. The plants selected for analysis, other than the control, each showed marked and characteristic symptoms of toxicity. It will be noted that definite increases in yield of total alkaloid occurred with excess molybdenum, and to a lesser degree with zinc.

It was readily verified in each deficiency experiment that only boron deficiency caused excessive branching of roots during growth. What was not anticipated was that just those excesses that increased root branching with respect to controls would also lead to increased production of total alkaloid. Alkaloid increase was found to differ in order of magnitude —Mo, Zn—just as did root branching. The secondary roots formed under the influence of the moderate toxicities used appeared to be entirely normal and made good growth. They differed in this respect from boron deficient roots. These tests are not considered to demonstrate specific differences in trace element toxicities, though these too may exist.

Comparison of the results on a percentage and quantity basis would indicate the unreliability of the former as a basis of comparison under some circumstances. There is no reason for assuming that concentration and quantity of alkaloid or even its relative partition between parts of the plant remain the same for any and all changes in environment.

Discussion

In all deficiency series only a shortage of boron led to an increase of alkaloid per plant. In the toxicity series excessive quantities of molybdenum or zinc had a similar effect. Each instance of enhanced alkaloid formation was accompanied by increased branching of roots, and vice versa. Values of alkaloid/root ratios, though based on yield instead of branching, displayed an unexpected uniformity in response. A causal relation between branching and alkaloid synthesis therefore seems probable.

Root branching in minus boron cultures differed from that caused by excess molybdenum or zinc. With boron deficiency the secondary roots attained a length of only several millimeters prior to blackening of the apical meristems. The secondary root development in the excess molybdenum and zinc cultures was normal in appearance.

The meaning of these alkaloid and root responses is not entirely clear. It is probable, however, that root metabolism is the major factor in alkaloid synthesis. Branching, of course, cannot take place without cell division and thus indicates the existence of an association between cell division and alkaloid synthesis. Dividing cells in root meristems are contiguous, however, with zones of cell elongation and differentiation, so all three types of growth might possibly be sites of nicotine formation. The sharply restricted growth in length of minus boron secondary roots (7) would indicate that cell elongation and differentiation may be feeble or non-existent.

Abnormalities in the tops should be considered in this connection. Boron deficiency led to death of the apical bud and many of the axillary buds. Enhanced alkaloid formation with excess molybdenum and zinc was unaccompanied by bud necrosis, but root branching was increased. Though additional study is needed, it appears probable that excessive root branching can in itself lead to increased alkaloid production without necrosis of apical and axillary buds.

Summary

Connecticut Broadleaf and Dixie Bright 101 tobacco were grown in water-culture and assayed for total alkaloid in green, oven- and air-dried samples. Boron deficiency led to large increases in total alkaloid per plant. Iron, zinc, copper, manganese, or molybdenum deficiencies resulted in decreased total alkaloid. Excesses of micronutrients led to decreased alkaloid content per plant with iron, copper, or manganese, and increased alkaloid content with excess molybdenum or zinc. Yield of plant with deficiency and excess was lower than that of the control in every trial. In each instance readily visible increases in root branching accompanied increased quantity of alkaloid.

Distribution of alkaloid was similar to that in healthy field-grown plants only in the controls. Deficiencies led to irregularity in that the root frequently had the lowest alkaloid content. The leaf always contained the largest quantity of alkaloid present in the plant, though its percentage content was sometimes lowest. Distribution of alkaloid was altered by drying.

Paper chromatography using tertiary amyl alcohol and color development with cyanogen bromide and *p*-amino-benzoic acid revealed the presence of characteristic alterations as compared with the control in quantities of nicotine, nornicotine, and anabasine in leaves of plants suffering from different deficiencies. Examination of the chromatograms with ultra-violet radiation showed the presence of unidentified non-alkaloidal fluorescent compounds not reacting with cyanogen bromide and *p*-amino-benzoic acid. These were also present in a manner characteristic of each of the micronutrient deficiencies. Oven-drying led to a decrease in non-alkaloidal fluorescent spots as compared with fresh samples, and air-drying to an increase.

The writers are indebted to Dr. K. R. Keller and Mr. W. H. Eoff of this Section for assistance in this work. Mr. Eoff conducted all the chemical determi-

nations and Dr. Keller subjected the data to statistical analysis. Of the conclusions listed in the first two paragraphs in the summary, eight were found to be statistically significant at the 1 % level and one at the 5 % level.

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THE FATE OF 2,4-DICHLOROPHENOXYACETIC ACID IN BEAN SEEDLINGS. I. RECOVERY OF 2,4-DICHLOROPHENOXYACETIC ACID AND ITS BREAKDOWN IN THE PLANT

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The early methods which were widely used in studying the transport of 2,4-dichlorophenoxyacetic acid (2,4-D) in bean seedlings depended on the growth response of the plants after the application of the auxin (4, 11, 18, 20, 21, 25, 26). Most auxins when applied to the primary leaf of a bean seedling will cause curvature of the elongating epicotyl and will inhibit the development of the terminal bud. These phenomena can be used for rough quantitative measurements of transport of the auxin, usually 2,4-D, but the results cannot be regarded as conclusive. This is because the evidence rests on growth responses which may themselves be influenced by the factors (other than 2,4-D) which are being varied. At best these criteria only measure the auxin movement from the leaf blade through the petiole and into the epicotyl. They cannot be used to determine the amount of 2,4-D that goes into non-growing zones such as the base of the hypocotyl, or into the roots. They do not allow of any distinction between uptake and translocation, nor can degradation of the 2,4-D be followed during the experiment.

Several attempts have been made to determine the extent of movement of herbicides by using 2,4-D containing C^{14} (3, 7, 15, 27) or 2,4-dichloro-5-I^{131} phenoxyacetic acid (2, 9, 19). There are obvious disadvantages to the use of labeled organic compounds for transport studies. Any organic compound when introduced into a plant is subject to possible degradation, and there is now considerable evidence in the literature to show that 2,4-D is readily broken down in test plants. Hansen and Buchholtz (10) and Evans and Smith (6) have presented evidence for the in vitro photooxidation of 2,4-D and *p*-chlorophenoxyacetic acid with the production of 2,4-dichlorophenol and *p*-chlorophenol. Evans and Smith (6) also have isolated a bacterium which actively destroys 2,4-D, again at the ether linkage. Splitting off of the side chain will in general lead to its oxidation. This probably accounts for the results of Weintraub and others which demonstrate that when 2,4-D with C^{14} in the side chain is given to plants, the C^{14} comes off in CO_2 (7, 15, 26) or is converted into numerous compounds other than 2,4-D (14, 15, 16, 28). Wain and his coworkers have demonstrated the formation of phenols from phenoxyalkylcarboxylic acids in the flax plant (24).

It follows that the presence of C^{14} in plant tissue does not indicate the intact labeled molecule that was originally applied. In some studies this has been realized, and the radioactive 2,4-D has been recovered from stems and identified by counter-current distribution (14) or by chromatography (8, 16, 17, 29); however, when this was done the greater part of the C^{14} was found either in two or three unknown compounds (8, 14, 16), or, when only very small amounts were applied, throughout numerous components of the tissue. It has also been suggested that the 2,4-D may enter complexes with other compounds. Holly (14) hydrolyzed his main ether-insoluble unknown product and obtained an organic acid which was not 2,4-D. However, Jaworski and Butts (16) state, without detailed data, that when their Unknown 1 was hydrolyzed and rechromatogrammed the decrease in its concentration was accompanied by a corre-

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sponding increase in the concentration of 2,4-D. Fang and Butts (8) hydrolyzed their Unknowns 1 and 3 which when rechromatogrammed gave 7 and 8 radioactive spots respectively. In each case, one of the spots had an R_f comparable to that of 2,4-D so that some 2,4-D may have entered a complex or been esterified. Similar results were obtained by Jaworski et al (17) when labeled 2,4-D was applied to etiolated bean seedlings. In no instance was the amount of 2,4-D set free by hydrolysis indicated, nor has it been definitely proved that the hydrolysate is really 2,4-D and not some other compound with a similar R_f.

In an investigation into the fate of a compound in plants one should be able (a) to detect the material with certainty and delicacy, (b) to distinguish between the original substance and its breakdown products, and (c) to determine the compound quantitatively in the various parts and thus to estimate the amount that has been absorbed by the tissues. This paper describes a method whereby the translocation and the disappearance of 2,4-D are followed simultaneously without depending on indirect criteria. It consists of extracting the herbicides quantitatively from various parts of the plant and bio-assaying the amounts recovered from each part. The classical slit-pea test which was used as the assay is specific for compounds with growing promoting activity. The test would respond to breakdown products of 2,4-D only if they were still capable of stimulating growth, and few, if any, of the probable breakdown products would have the characteristics required for activity. These characteristics have been the subject of an extensive literature (see 22 for reviews). It is known, for example, that phenols, whether or not halogenated are inactive or growth-inhibitory. Benzoic acids, with only two or three exceptions, are inactive (in particular 4-chloro- and 2,4-dichlorobenzoic acids are inactive) lactones are inactive or inhibitory, while arylacetic and propionic acids are usually active. A fortunate circumstance, which prompted the present study, is that 2,4-D is more active in this test than any other compound known, and is about ten times more active than the naturally occurring indoleacetic acid. The bioassay is also greatly superior to colorimetric tests which have been used for this purpose (5) but which are not specific for 2,4-D (1).

The Method

Preparation of Plants: Bean seeds (*Phaseolus vulgaris*, var. Dwarf Red Kidney) were soaked in shallow dishes of water for 6 hours and germinated between paper towels in a dark room at 25° C. When the radicles were approximately 10 cm long the seedlings were supported in the necks of 50 ml flasks containing tap water and kept in the dark for 24 hours longer. After this time the plants were transferred to light rooms maintained at 19° C and 75 to 80 % relative humidity where they received 1900 ft-c with a combination of fluorescent and incandescent light for 16 hours per day. These conditions were easily reproducible so that a constant supply of uniform plants was obtainable. Since the plants were grown in water, the total root system was readily available for extraction.

The 2,4-D was applied to the plants after they had been in the light rooms for 8 to 10 days. It was felt desirable to avoid the use of wetting agents so as to eliminate secondary effects due to additives. For this purpose, since absorption of the acid through the leaf surface is probably limited, another method of uptake had to be devised (fig 1). First, the cotyledons, terminal bud, and one primary leaf were removed to lessen the number of parts to be analyzed. A vial containing the solution to be taken up was fitted over a flap cut in the remaining primary leaf and held there with wads of cotton. The total volume of applied solution, 0.2 ml, was taken up in less than 12 hours when a fan was placed before the plants. The material left in the vial, plus that which could be washed from the flap, represented the amount not absorbed. By adding this amount to that extracted from the various parts of the plants all the recoverable auxin was accounted for, as will be shown below.

The Auxin Assay: The slit-pea test is the most convenient assay for the 2,4-D extracted from treated plants, both because it is relatively quick and simple, and because it is highly sensitive to 2,4-D and much less so to indole-acetic acid (IAA). As noted above, the relative activity of 2,4-D is from 8 to 12 times that of IAA in this test (22). The procedure, including the method of measurement, has been outlined by Thimann and Schneider (23). The apical 30 mm of the third internodes of etiolated Alaska pea seedlings 7 days old is used. Six sections, i.e., twelve curving halves, were placed in each dish of test solution. The test solutions were made up to 30 ml, of which 20 ml were placed in a Petri dish with the slit pea stem sections for testing and the remaining 10 ml used for the next dilution. In this way, serial dilutions of 1, 1/3, 1/10 etc. were obtained. For each test a reference curve (fig 2) was established from known concentrations of 2,4-D. The concentration of 2,4-D in the extracts was then established from those curvatures which fell on the linear part of the curve. Two or three such readings usually fell on this part of the curve. In all cases the figures presented represent the average of two or more trials run at different times but under similar conditions. Quantities as low as 1.5 μgm per sample could be detected. The test indicated no auxin activity in extracts from untreated plants even at the highest concentrations, so that the natural auxin was either destroyed in the extraction process or was present in quantities below the minimum detectable amount. Therefore, any auxin activity found in a test solution could be safely attributed to the applied material.

The Extraction Procedure: Considerable difficulty was encountered at first in recovering and accounting for large amounts of the applied 2,4-D from the treated bean seedlings. Simple water extracts were made but when the pea stem sections used in the

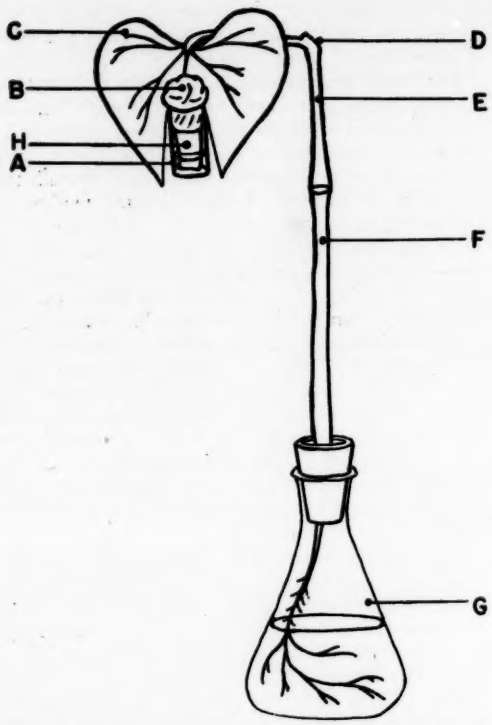
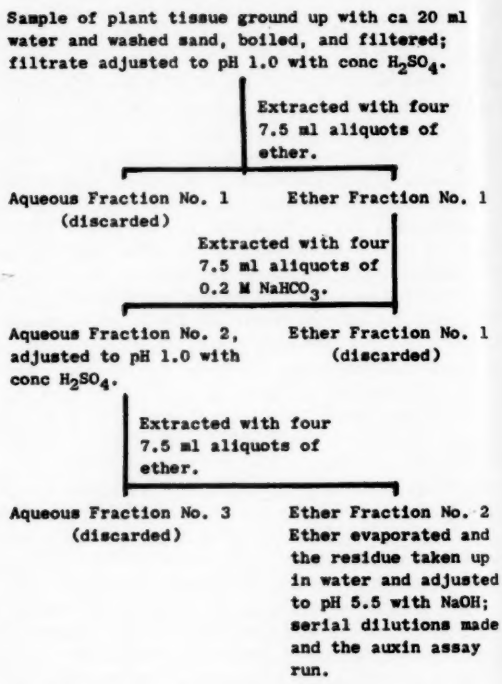


FIG. 1. Diagram showing the method of growing and applying 2,4-D to leaf of bean seedlings. A. Vial containing solution to be taken up. B. Cotton to hold vial in place. C. Primary leaf with flap inserted in the vial. D. The terminal bud and the other primary leaf have been removed. E. Epicotyl. F. Hypocotyl. G. Fifty ml flask containing tap-water. H. Flap.

assay were immersed in this they became flaccid so that reliable measurements could not be made. Accordingly, the plant material was ground in a mortar with water and washed sand, boiled to stop any destructive enzyme activity, and filtered. The filtrate was acidified to pH 1.0 and extracted with peroxide free diethyl ether. In this manner, the 2,4-D and other organic acids were separated from ether-insoluble and basic materials, but some neutral compounds would still be present in the extract. If, at this point, the ether was evaporated and the residue taken up in water and tested, still only about 30 to 40 % of the 2,4-D could be accounted for. Two explanations are possible for this apparently low yield; either much of the 2,4-D had been lost in the aqueous layer, or an inhibitor was present along with the 2,4-D. The latter explanation proved to be correct, for when the extract was further fractionated, up to 94 % recovery could be obtained (table I). The inhibiting agent was present in the neutral fraction.

Figure 2 shows the inhibition caused by the neutral ether-soluble components extracted from four bean leaves (ca 3 gm) when added to known amounts of 2,4-D. It was concluded that the apparently poor recovery was not due to faulty extraction but rather to inhibition of the pea stem curvatures by neutral compounds in the extracts.

The procedure finally adopted was as follows:


Sample of plant tissue ground up with ca 20 ml water and washed sand, boiled, and filtered; filtrate adjusted to pH 1.0 with conc H_2SO_4.
Extracted with four 7.5 ml aliquots of ether.
Aqueous Fraction No. 1 (discarded)
Ether Fraction No. 1
Extracted with four 7.5 ml aliquots of 0.2 M $NaHCO_3$.
Aqueous Fraction No. 2, adjusted to pH 1.0 with conc H_2SO_4.
Ether Fraction No. 1 (discarded)
Extracted with four 7.5 ml aliquots of ether.
Aqueous Fraction No. 3 (discarded)
Ether Fraction No. 2 Ether evaporated and the residue taken up in water and adjusted to pH 5.5 with NaOH; serial dilutions made and the auxin assay run.

Procedure for Extracting 2,4-D from Plant Tissues

EXPERIMENTAL

In the following experiment, 2,4-D was recovered from bean seedlings at different times after application using the above procedure. All the plants received 50 μgm of 2,4-D in 0.2 ml of water. Groups of 4 plants were harvested at 6, 24, 72 and 120 hours, and the leaves, stems and roots extracted separately. The amounts recovered from these parts, together with the percent total recovery at different times, are presented in table I. It is apparent that the recovery is very satisfactory at the beginning but decreases rapidly with time.

These data also show that the amount of 2,4-D found in the flap remained relatively constant with time so that disappearance in this part was negligible after 24 hours, in contrast to the condition in the remainder of the plant. A large number of trials, which will be described more fully in a second paper (13), confirmed that as the time between application and extraction increased the total amount of auxin recovered from the plants decreased. To obtain data for very short times the 2,4-D was applied to the base of cuttings where it was absorbed rapidly in the

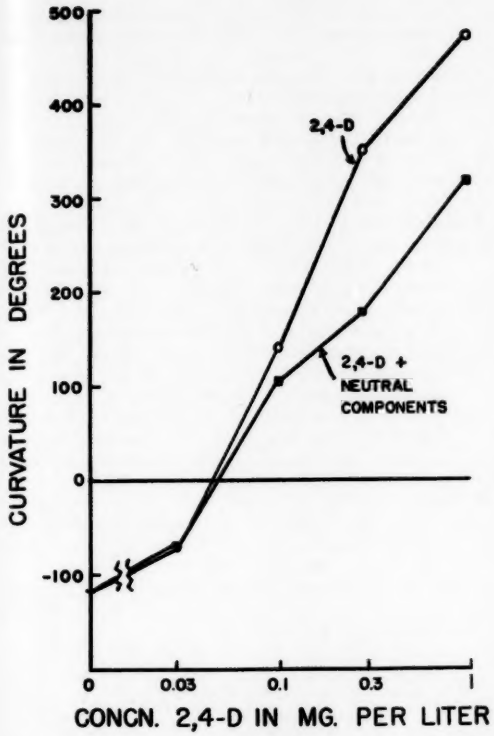


FIG. 2. Curvatures obtained with the pea test for serial dilutions of 2,4-D alone and for 2,4-D when added to neutral components of 4 bean leaves; pH 5.5 in both cases.

transpiration stream. These data are presented in figure 3 and further demonstrate the rapidity with which 2,4-D is either destroyed or converted to an ether-insoluble form in vivo. The amounts found in the flap and vial were excluded from these determinations, and the points represent the quantities recovered as percent of the total amount that moved beyond the flap or the immediate area of application. It is clear that when 50 μgm of 2,4-D were applied to these seedlings it disappeared almost completely in 4 or 5 days at 19° C.

TABLE II

PERCENT OF THE APPLIED 2,4-D RECOVERED FROM BEYOND THE FLAP AT 1 AND 5 DAYS AFTER APPLICATION IN THE LIGHT AND IN THE DARK

CONDITIONS	% RECOVERY	
	AFTER 1 DAY	AFTER 5 DAYS
Light	44	6
Dark	75	25

Fifty μgm 2,4-D were applied to the primary leaf of each of 4 seedlings; each figure is the mean of at least 2 trials.

Table II shows the percent total recovery of 2,4-D from bean seedlings at one and five days in light and in darkness. Although loss, presumably breakdown, was more rapid in the light, it also occurred with fair rapidity in the dark. It is clear that photoreactions do not account for the loss of all this auxin.

CONCLUSION

With the method described here it is possible to make direct measurements of the amount of 2,4-D that is translocated to any part of the plant. The method avoids the disadvantages mentioned above for other tests dependent on indirect criteria. It is believed to be specific for the applied synthetic auxin since no auxin was detected in untreated plants by this method. It was assumed that the 2,4-D did not induce the production of natural auxins in treated tissue. Experiments using this test to study some of the factors involved in the translocation of 2,4-D will be reported in part II (13), while its application to the transport of 2,4-D in a woody plant has been briefly presented elsewhere (12).

The data show the rapid disappearance of 2,4-D in vivo. Excluding the amounts in the flap and vial, less than 10 % of the 2,4-D that entered the remainder of the plant was recoverable at the end of 4 to 5 days. When 50 μgm of 2,4-D were applied to the bean seedlings about half of the auxin that left the treated area was broken down within just one day.

TABLE I

RECOVERY OF 2,4-D AT DIFFERENT TIMES FROM VARIOUS PARTS OF FOUR BEAN SEEDLINGS

TIME IN HRS	2,4-D RECOVERED (μGM)						% RECOVERY
	RESIDUE IN VIAL	FROM IMMERSED LEAF FLAP	FROM REMAINDER OF LEAF	IN STEM AND HYPOCOTYL	IN ROOTS	TOTAL	
6	150		34	4	0	188	94
24	15	43	29	34	0	121	60
72	0	51	3	17	0	71	35
120	0	45	2	7	0	54	27

Fifty μgm 2,4-D were applied to one primary leaf of each seedling. The other primary leaf was removed. A total of 200 μgm was applied.

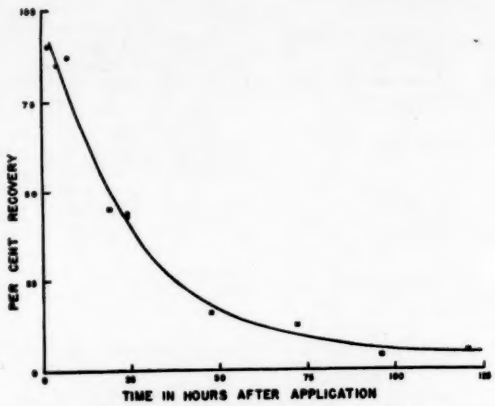


FIG. 3. Percent of the total 2,4-D applied (minus the quantities found in flap and vial) that were recovered from bean seedlings at various times after vial application, (open squares), and from cuttings of stem plus one primary leaf with uptake via transpiration stream, (closed circles). Fifty μgm 2,4-D were given in each case.

This agrees with the work where the intact labeled 2,4-D has been isolated and identified by countercurrent distribution (15), chromatography (8) or with a physiological test (29). As was mentioned above there is some indication that 2,4-D may enter a complex, and there is good evidence that 2,4-D is broken down in plant tissues if for no other reason than that up to 40 % of the total applied radioactivity cannot be accounted for at the end of 7 days (7). It is concluded that a large part of the 2,4-D lost in the present experiments is due to its degradation. The data serve to emphasize again the limitation to tracing the translocation of labeled 2,4-D simply by following the radioisotope.

An important advantage of the method described here is that the disappearance of physiologically active 2,4-D can be easily followed together with its translocation.

SUMMARY

A method for the extraction and bioassay of 2,4-D from treated plants is described. It is shown that recovery from bean seedlings is quantitative but that freely extractable 2,4-D rapidly disappears in the plant. This loss, presumably breakdown, is considerably faster in light than in darkness but even in the dark three-quarters of the absorbed 2,4-D disappears in 5 days. The advantages of the method over those based on indirect criteria are discussed.

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THE INTRACELLULAR LOCATION OF PHOSPHORYLASE IN TOBACCO (NICOTIANA TABACUM L.) [1,2]

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Hanes (6, 7) first identified the enzyme phosphorylase in preparations of pea seeds and potatoes. The enzyme catalyzes the reaction glucose-1-phosphate (G-1-P) $\rightleftharpoons$ starch + phosphate, and Hanes predicted that the location of this enzyme would be the plastid of the plant. Yin and Sun (15) presented a histological mehod for identifying phosphorylase activity by the end product starch, and Yin and Sun (17) presented evidence for the location of phosphorylase in the plastids of several species of plants.

Using the above histological method on wheat, bean, and sunflower, Shaw (10) also found phosphorylase activity to be within the chloroplast, or associated with it, and he reviewed the many sources of error which leave the method open to criticism. A principal criticism is that plasmolysis could not be demonstrated in cells exhibiting polysaccharide formation. There is no certainty then that in these injured cells the enzyme is to be found in its "normal" location.

Dyar (5) used the histological method on root tips of pea seedlings, and she found activity in the nucleus and unspecified areas of the cytoplasm.[3] Stocking (11) used the histological method and a method based on the differential centrifugation of a leaf brei and could find no evidence that phosphorylase was within the plastid rather than the cytoplasm. The investigation reported here was made in part to find if the problem of phosphorylase in the plant cell could be resolved by the application of other methods.

Investigations by the author, based on quantitative methods, have shown daily and seasonal variations in the level of activity of phosphorylase, and have shown a considerable reduction in the level of activity when tobacco plants were starved by placing them in the dark. Since the principal test for activity used has been the qualitative formation of starch, previous workers have generally thought it advisable to use materials in which starch was not initially present. The low level of activity in such tissues may yield results which do not reflect conditions in a healthy plant. Hence some of the techniques here reported were designed to obtain data about the intracellular location of phosphorylase in tissues in which starch was present and being actively metabolized.

MATERIALS AND PREPARATIONS

Leaves of tobacco (*Nicotiana Tabacum* L.), stripped of heavy veins, were used as the plant material. G-1-P was obtained from Nutritional Biochemicals Company and its solutions were prepared using the method of Sumner et al (13). A 2% solution was made and diluted with an equal volume of 0.01 M maleic acid buffer pH 6.2 to give a 1% solution. Buffers were prepared using the acid and sodium salt. As sodium may increase permeability this is of concern in interpreting the results.

Some preparations were made by grinding fresh leaf tissue in a mortar with a small amount of phosphate free quartz sand and with 1 ml of solution as indicated per gm of tissue. The preparation was washed down with an additional 2 ml of solution per

[1] Revised manuscript received May 23, 1956.

[2] Part of this work has been done in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Cornell University.

[3] Cytoplasm is used in this paper to denote protoplasm other than the nucleus or plastids.

gm of tissue. Other preparations were made by grinding leaf tissue in a Waring blendor with 3 ml per gm of either 12 % sucrose solution in pH 6.2 maleic buffer, or 60 % saturated neutral ammonium sulfate solution. The crude preparations were filtered through a facial tissue supported on cheesecloth.

Methods and Results

In these experiments phosphorylase activity was measured in terms of the free inorganic phosphate released when the plant preparations were incubated with G-1-P under the conditions stated. One ml of preparation was placed in a test tube with 1 ml of 0.01 M maleic acid buffer, pH 6.2; 1 ml of a 1 % soluble starch solution as a primer (6) and 1 ml of 0.02 M NaF as an inhibitor of possible phosphatase activity. These were brought to temperature (35° C) in a water bath and the reaction was started by adding 1 ml of 1 % G-1-P. At the end of the experiment the reaction was stopped by adding 1 ml of 10 % trichloroacetic acid (TCA). In the controls the order of adding the TCA and G-1-P was reversed. The 6 ml of preparation resulting were filtered and 3 ml were tested for phosphate by the use of Sumner's (12) modification of the phospho-molybdate test. The amount of phosphate was determined colorimetrically by reading percent transmission in a Coleman Jr. Model 6A spectrophotometer, at 680 mμ. Either density or micrograms of phosphorus was then determined from tables. Since Beer's Law holds in the range of values obtained, the statistical comparisons were calculated on the basis of the optical density.

Analysis showed that no significant breakdown of G-1-P occurred in 4 hours at room temperature after the addition of TCA. After the addition of H_2SO_4 in the phosphomolybdate test, however, acid hydrolysis of G-1-P began at once and so all readings were made between 1.5 to 2 minutes after adding the sulfuric acid.

The time of incubation was from 1/2 hour with preparations of high activity up to 1 hour when the tobacco leaves showed low phosphorylase activity. As comparisons were made within the experiments, such variation between experiments was considered allowable.

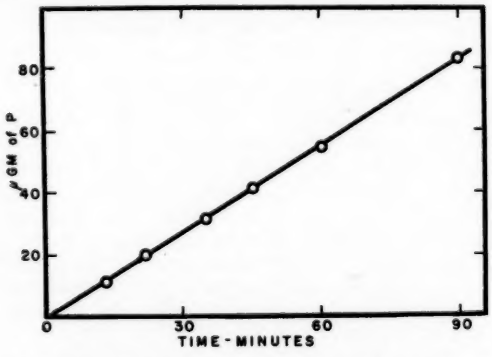

Fig. 1. Hydrolysis of G-1-P by phosphorylase. About 1190 μgm of P were supplied as G-1-P. The hydrolysis is a straight line reaction for the range of values used (up to 100 μgm hydrolyzed).

Table I

Relative Phosphorylase Activity of Residue (R) and Supernatant (S) of Centrifuged Tobacco Leaf Brei

Ml of residue		Activity */ml			
		Residue			
Initial	Resuspended	Resuspended	Initial	Supernatant, initial	Ratio of activity, (R/S)
2	12	26.3	157	37	4/1
1	10	10	120	17	7/1
0.5	10	7.4	148	5	30/1

* As μgm of P hydrolyzed from G-1-P in 30 min at 35° C.

One ml of 1 % G-1-P contains 1.19 mg of inorganic phosphorus. The greatest amount hydrolyzed in any single experiment was less than 100 μgm. The reaction was found to be a straight line for these values (fig 1).

Other experiments showed no deterioration in the phosphorylase activity of the preparation during the first 4 hours at room temperature. For this reason low temperatures were not used in making the preparations; however, the experiments were executed with promptness.

The first group of experiments was run with the following hypothesis as a basis. The enzyme phosphorylase is water soluble. When ground preparations are centrifuged the phosphorylase will be found in the supernatant in any case, since a large percentage of the plastids are ruptured in grinding. If the phosphorylase is normally located in the cytoplasm, then that portion of the centrifuge tube containing the residue should have less phosphorylase per unit volume, as phosphorylase would be displaced by the solid residue. On the other hand, if the phosphorylase is normally located within the plastid, then the residue should have the higher concentration of phosphorylase, its value being proportional to the number of uninjured chloroplasts.

Preparations were made by grinding leaf tissue in a mortar with pH 6.2, 0.01 M maleic buffer and fine sand, as described, and the filtered brei was centrifuged. The results in all cases showed a higher concentration of phosphorylase activity in the green pellet of the residue. The greatest total amount of activity was invariably in the supernatant, but the highest concentration was in the residue. Examples of data are given from three such experiments in table I.

In the first of the experiments which are described, ground and filtered leaf material was centrifuged lightly (250 × g for 3 min) and the residue which was principally starch was discarded. The supernatant was centrifuged for 20 minutes at 800 × g and 2 ml of

chloroplastic residue was obtained. The 2 ml of residue was brought to 12 ml by adding 10 ml of buffer and was then re-ground in a Potter-Elvehjem type homogenizer. One ml of the first supernatant hydrolyzed 37 μgm of phosphorus in 30 minutes. One ml of the second supernatant (derived from 1/6 ml of chloroplastic residue) hydrolyzed 26.3 μgm of phosphorus in the same time. On a ml for ml basis then the ratio of the activity of the supernatant and of the chloroplastic residues was 37/157 or about 1/4.

In a second example the ratio of activities was about 1/7. These two examples represent the range of differences obtained in experiments of this sort. It was found that regrinding the residue was not necessary and that shaking the residue in buffer to resuspend it gave the same values as regrinding. An effort was made to improve these first figures by using gentler techniques. Since the sodium ion affects permeability, no buffer was used. A 10 % sucrose solution was deionized by passing over amberlite resins. The resulting neutral solution was chilled in an ice bath. Cell contents were released by gently tearing tobacco leaves under the surface of the solution. One-half ml of green residue was obtained by centrifugation, and this was re-suspended in 10 ml of buffer. Total activity was still greater in the supernatant. Two and one half times the activity was contained in the 40 ml of supernatant as in the 0.5 ml of residue. In this instance the ratio was widened and the concentration of phosphorylase activity was, however, thirty times as great in the residue.

These results suggesting a concentration of phosphorylase in the chloroplast are at variance with the conclusions of Stocking (11). At this point in his work the author had the opportunity of comparing his data with that of Dr. Stocking and of discussing it with him. A comparison showed that the principal discrepancy between the works lay in Stocking's use of the plastid fraction of leaves which had been dark-starved to remove starch. In preliminary experiments the author had found that dark starvation reduced phosphorylase activity to low levels, and had so designed his experiments as to use leaf material gorged with stored starch. This difference in material was examined as a possible source of the differences of result. Typical and comparable leaves were taken at 10:00 A.M. from a tobacco plant which had its starch depleted by seven days of dark starvation and one growing in the greenhouse in the summer sun. Leaf (1.5 gm) tissue was ground in 5 ml of buffer and washed with 10 ml, filtered and centrifuged. The 0.2-ml pellet was suspended in 4 ml and activity was determined over a 55-minute period at 35° C. The results of a typical experiment are given in table II. These results indicate that at least some phosphorylase activity is primarily in the chloroplast in a plant containing starch and actively synthesizing it, but that it appears in the cytoplasm of a plant which does not contain starch. In addition it may be noted that the total activity is much greater in the plant kept under normal conditions.

TABLE II

RELATIVE PHOSPHORYLASE ACTIVITY OF SUPERNATANT (S) AND RESIDUE (R) OF CENTRIFUGED TOBACCO LEAF BREI FROM FULL SUN AND DARK STARVED LEAVES

TREATMENT	CORRECTED ACTIVITY*/ML R	ACTIVITY*/ML S	RATIO, (R/S)
Sun	290	23	13/1
Dark, starved	nil	12	0

* As μgm of P hydrolyzed from G-1-P in 55 min at 35° C.

TABLE III

RATIOS OF PHOSPHORYLASE ACTIVITIES* IN PLASTID CONTAINING RESIDUES (R) AND SUPERNATANTS (S) DERIVED FROM CENTRIFUGED TOBACCO LEAVES GROUND BY VARIOUS METHODS

TREATMENT	RATIO, (R/S)
Cut into 12 % deionized sucrose with razor blade	37/1
Bruised with pestle in 12 % deionized sucrose	9/1
Ground in mortar with sand and 12 % sucrose in maleic buffer	6/1
Blendorized 5 sec in 12 % deionized sucrose	8/1
Blendorized 45 sec in maleic buffer	5/1

* Activities based on μgm of P hydrolyzed from G-1-P/ml of preparation.

Dark starved vs full sun plants were also compared using more and less drastic methods of "grinding." The activity of dark starved leaves was in the supernatant. In the leaves from the full sun plants, the phosphorus hydrolyzed per unit volume of pellet was always greater than that per unit of supernatant. Data from full sun leaves is presented in table III. The method of "grinding" is given, with the ratio of the concentration of phosphorylase in the pellet to that in the supernatant.

The second group of experiments was based on the following hypothesis. If the phosphorylase can be kept in situ by precipitation and if differential centrifugation can produce a series of pellets having different proportions of various cell fractions, then an analysis of chlorophyll content (chloroplasts), DNA (nucleus) or total protein (cytoplasm) can be compared to an analysis of phosphorylase activity and a correlation sought.

The final procedure which was developed did not include the analysis for DNA as the amount present in leaf tissues is low and insufficient for the methods of analysis that were available. The leaf tissue was taken either from dark starved plants or those growing in the greenhouse and was ground in the blendor with either 12 % sucrose or neutral 60 % saturated $(NH_4)_2SO_4$. The filtered brei was placed in centrifuge tubes and the centrifuge was run at varying speeds for varying lengths of time to obtain various

degrees of sedimentation. Ten such tubes were centrifuged for each experiment. The tubes contained 12 ml initially and after centrifugation the green pellet was resuspended in 4 ml of its own supernatant. Some of the supernatants were included as preparations. One ml of preparation was used to test phosphorylase activity by the method described, the incubation period being 1 hour at 35° C. To a second 1-ml aliquot was added 10 ml of alcohol. The tubes were brought up to the boiling point of the alcohol mixture in a hot water bath to facilitate coagulation of the protein and extraction of the chlorophyll. The coagulum was centrifuged down and the optical density of the supernatant was obtained spectrophotometrically at 664 mμ to compare the relative amounts of chlorophyll. When the residue was from one of the groups where the leaves had been ground in ammonium sulfate solution, the pellet was given an additional washing in 3 to 4 ml of 5 % TCA solution to remove excess salts. After centrifugation the pellet was treated the same as the alcohol precipitated pellet from the groups where the leaves were ground in sucrose. The pellets were suspended in 10 ml of 3.3 *N* NaOH solution and heated at 80° C for 2 to 3 hours to dissolve the pellet. The volume of the tubes was brought back to 10 ml if evaporation had occurred and 0.4 ml of 20 % copper sulfate pentahydrate solution was added to each tube and the tube shaken. The tubes were centrifuged and the optical density of the supernatant was obtained at 530 mμ to give relative amounts of protein. For each group of ten tubes the coefficient of correlation was calculated between hydrolyzed P and chlorophyll content, between hydrolyzed P and protein, and between protein and chlorophyll content. The results in table IV present the one poorest correlation from each group. From 2 to 5 groups were run for each condition. Further repetitions were not made because of the great consistency of the results, and because these results contributed little to the problem under consideration.

In the preparations made with ammonium sulfate, all three factors, protein, chlorophyll content, and phosphorylase activity were highly correlated. This indicates that the three are distributed homogeneously in the same particles of the solid phase so that an aliquot samples all three uniformly.

In the preparations made with sucrose the high correlation between chlorophyll content and protein indicates again that in an aliquot the solid matter contains a homogeneous mixture of these two. The relatively poor correlations between phosphorylase and the other two factors indicates its distribution largely in the soluble fraction of these preparations. The positive correlation of P to C in sun grown plants and the negative correlation in dark starved plants was consistent and may be interpreted as bearing out the data from the other methods; i.e., the negative value of r for dark starved plants may again be explained by the displacement of the solution, having uniform phosphorylase content, by the solid fraction containing the chlorophyll, water insoluble protein and no phosphorylase. The value of r would be positive for sun plants due to some of the phosphorylase being associated with the solid (plastid) fraction.

Inasmuch as material in solution is evenly distributed, correlation should arise out of the differences in the solid fraction. This was checked in one experiment by centrifuging an extra aliquot from each sample and determining protein separately for the supernatant and the residue. The data are given in table V. They were obtained from a run using full sun plants ground in sucrose. The data effectively support the deduction. The negative correlations arise

TABLE V

COEFFICIENT OF CORRELATION BETWEEN PHOSPHORYLASE AND PROTEIN AND BETWEEN CHLOROPHYLL AND PROTEIN IN 1 ML OF A TOTAL TOBACCO LEAF BREI AND IN THE RESIDUE AND SUPERNATANT FROM 1 ML OF THE CENTRIFUGED BREI

FRACTION	COEFFICIENT OF CORRELATION, r	
	PHOSPHORYLASE/PROTEIN	CHLOROPHYLL/PROTEIN
Protein of uncentrifuged brei	0.905	0.963
Protein of residue	0.895	0.943
Protein of supernatant	−0.598	−0.680

TABLE IV

COEFFICIENT OF CORRELATION BETWEEN PHOSPHORYLASE ACTIVITY AND CHLOROPHYLL CONTENT, PHOSPHORYLASE ACTIVITY AND PROTEIN CONTENT, AND BETWEEN CHLOROPHYLL AND PROTEIN CONTENTS IN TOBACCO LEAVES GROUND IN VARIOUS MEDIA FROM PLANTS IN FULL SUN OR DARK STARVED

TREATMENT	GRINDING MEDIUM	COEFFICIENT OF CORRELATION, r *		
		PHOSPHORYLASE/CHLOROPHYLL	PHOSPHORYLASE/PROTEIN	CHLOROPHYLL/PROTEIN
Full sun	12 % Sucrose	0.339	0.337	0.910
Full sun	60 % $(NH_4)_2SO_4$	0.964	0.967	0.883
Dark starved	12 % Sucrose	−0.389	−0.155	0.948
Dark starved	60 % $(NH_4)_2SO_4$	0.915	0.989	0.983

* At 1 % level of significance, r = 0.765.

because as there is more residue, there is less supernatant in one ml.

Discussion

The experimental evidence presented here indicates that both the plastid and the cytoplasm may be normal sites of the enzyme phosphorylase. Although the evidence for phosphorylase in the plastid is based on normal plants and that for phosphorylase in the cytoplasm on dark starved plants, the data do not preclude the possibility that phosphorylase may occur at both sites under either condition.

The data showing phosphorylase distribution may be subject to the following interpretation. In centrifuging, a starch pellet is thrown down beneath the plastid material. Even though this is not used, the remaining material is heavily contaminated with free starch. The association of an enzyme with its substrate is well known and Baum and Gilbert (2) have used this relation to purify phosphorylase by adsorbing it on amylose. The concentration of phosphorylase in the pellet may, therefore, be due to adsorbtion on the starch.

When the phosphorylase content of the plastid fraction is increased by the use of gentle techniques, the increase may result because more starch is retained in the green pellet within the unruptured plastids.

The attraction between starch and phosphorylase provides then an adequate explanation of the data presented here. It fails however to explain why—if phosphorylase is of general distribution—starch is found almost exclusively in the plastids.

Stocking (11) has set forth the 4 requirements for amylose formation.

1. pH about 6
2. Active phosphorylase
3. α-D-glucose-1-phosphate as substrate
4. Starter—an α,1-4 glucoside chain to prime the reaction.

1) The range of pH over which phosphorylase is active is wide enough so that a difference of pH between the plastid and cytoplasm would not account for activity at only one site.

2) An inhibition system can be postulated. Known elements are available, as β-amylose inhibits phosphorylase (9) and is itself inhibited by sucrose (4). Stocking has considered inhibition however and shows that it is an unlikely mechanism.

3) The plastid may well be the principal site of starch formation because it is the exclusive site of the formation of the substrate G-1-P. In Calvin's (3) experiments he found G-1-P to be labeled with C-14 after 5 seconds of photosynthesis in $C^{14}O_2$ medium. G-1-P is not reported as participating in reactions in higher plants other than in its equilibrium reactions with G-6-P, and with amylose and phosphate.

Arnon, Whatley and Allen (1) have recently presented evidence for a hypothesis that the chloroplast is a complete synthesizing unit, using the energy of sunlight to manufacture its end products without intermediate cytoplasmic steps. The formation of G-1-P and hence starch in the plastid would be consistent with such a hypothesis.

Figs. 2 and 3. Longitudinal sections of promeristem of tobacco shoot apex showing results of Yin's histological test for phosphorylase. P—Plastid not forming polysaccharide. S—Plastid which has formed starch. A—Amylose network. Fig. 2 (*left*) Incubated with G-1-P. A blue-brown staining polysaccharide has formed within the plastids of the endodermis or starch sheath. Fig. 3 (*right*). Incubated with G-1-P and soluble starch solution. An additional lavender-blue staining network of polysaccharide has formed in the cytoplasm generally.

This explanation still fails to answer one question. If amylose production is limited by the location of G-1-P rather than the location of phosphorylase why does not amylose appear generally when G-1-P is supplied in Yin's test? It often does. In an early report Yin (16) found polysaccharide formation in loci in the cytoplasm. He concluded in a later paper (17) that phosphorylase was exclusively in the plastid.

Stocking (11) reports cytoplasmic starch in leaf sections prepared by using Yin's histological method.

Shaw (10) reported "the most common result was the formation of irregular masses of polysaccharide in the cytoplasmic matrix around the chloroplast." Shaw considers this due to diffusion of polysaccharide out of the plastids.

Paech and Krech (8) find small kernels forming outside of the plastids in their immediate vicinity. They consider that the phosphorylase has leaked out of the plastids.

Dyar (5) reports starch formation in unspecified areas of the cytoplasm and in the nucleus.

The histological test has then yielded results showing the formation of polysaccharide in the cytoplasm supplied with G-1-P. The interpretations of the results have varied. Figures 2 and 3 show some results of the use of the histological test in this laboratory. Londitudinal free-hand sections of the same tobacco stem apex are shown after incubation for 4 hours in G-1-P. Soluble starch solution was added to the G-1-P medium of the one section (fig 3). Figure 2 shows the results of incubation in G-1-P alone. A brownish blue staining (IKI) polysaccharide has formed in the plastids of the endodermis or starch sheath. In figure 3 with soluble starch primer present

the same formation of polysaccharide in the plastids of the endodermis has taken place, and in addition a cytoplasmic polysaccharide has formed. A loose network of bright lavender-blue staining polysaccharide occurs in the cytoplasm of the cells generally.

4) This brings us to a consideration of the requirement for primer. Starch may form only in the plastid in vivo because this is the sole site of primer. This seems unlikely however. Whelan and Bailey (14) have found that α,1-4, tri glucoside units are sufficient to prime the reaction. Polysaccharide of such chain length is readily diffusible and occurs as a product of α-amylose hydrolysis along with diffusible chains of 6 glucose units in length.

Of the four possibilities the one which best fits the experimental evidence is that, that the plastid is the exclusive side of the formation of G-1-P substrate. In metabolically active cells the phosphorylase may become concentrated in the plastids because of the adsorbtion (or other chemical binding) of the enzyme on the starch surface and because of the protective effect of substrate in reducing catabolic destruction of the enzyme. When G-1-P is supplied to the whole cell, polysaccharide may or may not form in the cytoplasm in the period of observation. If either primer or enzyme are in low concentrations in the cytoplasm the rate of synthesis may be so low that stainable polysaccharide is not formed within the duration of the experiment.

Shaw (10) found that he obtained his results only during the summer period. This laboratory has found that seasonal factors affect phosphorylase activity. The work described here was done on vegetative plants, for it appears that once the flower is induced, even prolonged dark starvation will not remove all the starch from leaves. As we are better able to understand phosphorylase it should provide a tool for investigating other plant reactions.

Summary

Evidence is offered that phosphorylase may occur in both the plastid and in the cytoplasm surrounding it. The data indicate a concentration of phosphorylase with the plastids of leaves actively synthesizing starch and a concentration in the cytoplasm of leaves dark starved to remove starch.

The hypothesis is offered that starch is normally found in the plastids because that is the site of formation of the G-1-P substrate.

The author is pleased to acknowledge the advice and assistance generously given by Dr. D. G. Clark of Cornell University.

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SOME STUDIES OF THE ADSORPTION OF ZINC SULFATE IN THOMPSON SEEDLESS GRAPE CANES[1]

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Little leaf of grapevines is corrected by applying zinc to the vine (1, 2, 3, 4, 5, 6). Different methods have been used to supply zinc to the vine; metal points have been driven into the trunk, zinc salts placed in holes bored in the trunk and in the soil, zinc salts in solution injected into the trunks, solutions dabbed or sprayed on the freshly cut pruning wounds and zinc salts sprayed on the foliage. All of these methods have given variable results and appear to have their limitations. Many soils seem to fix zinc so that the vines do not pick it up through the roots. Spray applications to the foliage have given erratic results in different parts of California and the successful application to pruning wounds has been limited essentially to the spur-pruned varieties. Furthermore, the application of zinc sulfate in solution at 228 grams per liter to the freshly cut pruning wounds has in some seasons caused considerable damage to the buds and spurs. Weaker solutions of zinc sulfate have been applied to freshly cut pruning wounds of Muscat of Alexandria in December and January with good response in yield and little damage to the spurs (6), but the mechanism of zinc movement into the spur through the pruning cut is not understood.

There are many acres of cane-pruned grapevines in California, especially of the *Vitis vinifera* var. Thompson Seedless, that would apparently respond to zinc if effective means of application were found. Field tests with sprays have given inconsistent results and treating freshly cut pruning wounds of these cane-pruned varieties has not given satisfactory response.

Knowledge of the movement of zinc within the vine and factors involved in the penetration of zinc salts into the pruning wounds should prove helpful in treating zinc deficiency. Radioactive Zn^{65} is especially suited for tracer studies of the movement of zinc. It emits both β and γ radiations and has a relatively long half life of 250 days. The long half life makes it unnecessary to correct for decay time for experiments conducted over short periods of time.

GENERAL METHODS

Radioactive zinc sulfate ($Zn^{65}SO_4$) was prepared from old copper deflection plates from a cyclotron by etching the plates with nitric acid and separating the minerals with H_2S in the usual way. Two different solutions were used, each prepared at different times, one contained about 0.5 mc of activity per 100 ml of solution and the other about 10 mc. The activity of each solution was determined in terms of the γ radiation which comprises the major part of the radiation.

Dormant canes of the Thompson Seedless grapevines, about 1.6 to 2.3 meters long with no side branches, were cut from the vines and used in these experiments. Glass tubes of about the same diameter as the cane were fitted to the base ends with a short rubber collar. The canes were suspended vertically with the base up. Test solutions, called flushing solutions, were placed in the glass tube attached to the base. The tips of the canes were cut back to the desired length to allow the flow of solutions through the canes from base to tip. Ten ml of solution generally passed through 1.5 m of cane within 10 to 20 minutes, though in some cases it took much longer.

The radioactivity of canes and cane sections was determined by means of a Geiger-Müller tube with a Tracer Laboratory Autoscaler. When the counts per minute (cpm) in samples were very high corrections were made for the loss of counts occasioned by the coincidence of ionization occurring during the "dead time" of the Geiger tube. The corrections for loss by coincidence counting were determined by the usual method of making up a set of planchets of different counting rates, and cutting the planchets in half. The counts for each half were determined separately and then together and curves drawn for the loss of counts for the set. The counts for the grape cane sections in these studies were then corrected by the equation, $N_t = N_o\ (1 + 6.25 \times 10^{-6}\ N_o)$ where N_o is the observed counts and N_t the counts corrected for coincidence losses for the particular tube used. Corrections were made for all samples having counts of over 5000 per minute. The loss at 25,000 cpm, for example, amounts to about 16 %. The loss in counts at 5000 cpm was about twice as great as the statistical uncertainties, i.e., about 3 %.

RESEARCH

EXPERIMENT 1: A dormant cane was prepared and the amount of water in the vessels of a 1.5-m section was determined by sucking the water out into a trap with a vacuum. This section of grape cane yielded 3.5 ml of water by this method. A 1-ml aliquot of the low activity $Zn^{65}SO_4$ solution was diluted to 4 ml with distilled water and placed in the glass tubing attached to the base of another cane. This amount of solution would just approximately displace the solution in the cane. The tip of this cane was cut off at 1.62 m and the solution allowed to flow through it. The exudate, 3.8 ml in all, was collected in 1 ml lots and examined for radiation. The cane was then pushed through a cylindrical sample chamber having 5.7 cm thick lead walls for radioactivity measurements (Tracer Laboratory). The Geiger-Müller tube was an end-on type having a thin mica window of 2.54 cm diameter (Tracer Laboratory), and of about 1.8 mg/cm^2. The radioactivity of the cane was determined by exposing a part of the cane at 10-cm intervals to the Geiger-Müller tube and recording the counts. Due to the thickness of the lead walls and the nearness of the cane to the tube window (about 6 mm), the radiation recorded was principally that from a small section of

[1] Revised manuscript received June 2, 1956.

cane directly under the Geiger tube window. There was no radioactivity above normal background measurable in the exudate and in this cane most of the activity was confined to the first 50 cm from the base where the solution was introduced. The base of this same cane was then attached to a supply of distilled water with approximately 5 m of pressure. The exudate at the tip of the cane was collected in 1-ml lots for the first 6 ml and then in 5-ml lots for the next 25 ml. No radiation above background was found in any of the samples. After this, additional water was forced through the cane until a total of 2385 ml was finally passed through this cane. The entire lot of water was concentrated by boiling to about 10 ml. This concentrate gave a radiation count of 560 per minute above background showing that some of the radioactivity had been washed out of the cane by water. Radiations from the cane, as indicated by the counter, weakened and the destribution shifted slightly in the direction of flow in the cane, but showed the same general pattern.

Experiment 2: Another cane was prepared and 10 ml of water containing 1 ml of the weaker $Zn^{65}SO_4$ solution was allowed to pass through it. The radiation along the cane was determined as before. Most of the activity was found in the first 30 cm of this cane (fig 1 a). It was then flushed with 100 ml of distilled water and the distribution of activity determined (fig 1 b). The cane was then flushed with 10 ml of a 10 % solution of non-radioactive $ZnSO_4$, and the distribution of radioactivity again determined (fig 1 c). Finally it was flushed with an additional 480 ml of the 10 % solution of non-radioactive $ZnSO_4$ and the radiation along the cane determined (fig 1 d). Figure 1 shows that after passing the initial solution through the cane, most of the radioactivity was fixed in the first 30 cm. Flushing with 100 ml of water reduced the activity in the first 15 cm and increased slightly the activity at subsequent points along the cane. Similar and progressive results were obtained by flushing with the 10 ml of 10 % $ZnSO_4$ solution. After flushing the cane with the additional 480 ml of $ZnSO_4$ the activity in the first 25 cm was greatly reduced, but little or no change occurred in the rest of the cane. The radioactivity found in the 480 ml of flushing solution after passing through the cane accounted for the decrease of activity in the cane. It is probable that the 10 ml lot of 10 % non-radioactive $ZnSO_4$ flushing solution supplied sufficient salt to saturate the remainder of the cane and therefore the radioactive zinc displaced by subsequent flushings did not redeposit further along in amounts detectable by the methods used.

pH Studies: Additional experiments indicated that the pH of the flushing solution affected the deposit of radioactivity along the cane. Therefore, a series of solutions of $Zn^{65}SO_4$ was prepared using 1 ml of the low activity zinc in each. These aliquots were diluted to about 10 ml with distilled water and adjusted to the following pH values with HCl and NaOH: 0.55, 1.45, 2.33, and 4.55. Each solution was

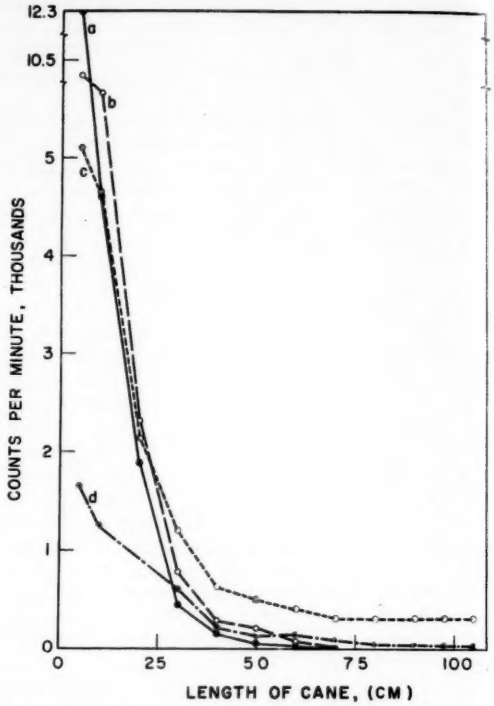

Fig. 1. Number of radiation cpm at 10-cm intervals showing the distribution of Zn^{65} along a grape cane: a) after passing 10 ml of a weak $Zn^{65}SO_4$ solution through the vessels from base to tip; b) after flushing with 100 ml of water; c) after flushing with 10 ml of a non-radioactive 10 % $ZnSO_4$ solution; and d) after flushing with an additional 480 ml of non-radioactive 10 % $ZnSO_4$ solution.

then passed through a different grape cane. The pH and radioactivity of each exudate were determined. Canes were cut into 5-cm lengths and the radioactivity of the piece of each determined. The distribution of radioactivity from the cane receiving the solution adjusted to pH 4.55 resembled very much that in figure 1 where most of the radioactivity was deposited along the first 25 to 30 cm of the cane. Zinc^{65} was more evenly distributed in canes which received solutions with pH of 2.3 and less. The pH of the exudate from the canes receiving the solution adjusted to pH 4.55 was pH 6.2; that for 2.33 was 6.1; that for 1.45 was 5.8, and that for 0.55 was 3.4. The radiations from these exudates were respectively 0, 0, 5080, and 6580 cpm above background.

The experiment was repeated using flushing solutions adjusted to pH values 1.0, 2.0, 3.1, 4.0, and 5.0. After flushing, the canes were left hanging overnight, then in order that more accurate readings could be made the canes were cut into pieces 2.54 cm long and the radioactivity of each piece was determined and is shown plotted in figure 2. The types of activity-dis-

tribution curves obtained and the levels of activity are similar to those in the previous experiment.

From the solution adjusted to pH 1.0, the cane adsorbed very little radioactivity, but as the pH values of the solutions increased, the activity adsorbed by the cane also increased, especially in the first part of the cane. At pH values of 4.0 and 5.0 the first few cm of cane adsorbed large quantities of radioactive zinc. After this initial uptake the remainder of the cane adsorbed more uniformly from the flushing solutions, thus indicating some change in the flushing solution within the cane that prevented adsorption of the radioactivity.

Experiments similar to that just described were run using $Zn^{65}SO_4$ solution with a much higher specific activity. Solutions at pH values 1.0, 1.2, 2.9, 3.9, and about 5.4, or just below the precipitation point for zinc hydroxide, were prepared and passed similarly through dormant canes of Thompson Seedless. After standing overnight, the canes were cut into 2.54-cm sections from base to tip. The radioactivity of each piece of cane was determined and plotted as shown in figure 3.

The distribution of activity in the first few cm of the cane was similar to that obtained in previously conducted experiments except that at the lower pH values the curves are steeper in the first 25 cm and a much higher order of activity continued through the cane. The exudate solutions all ran fairly high in activity.

All of the canes in this pH series were apparently more nearly saturated with radioactivity than were those canes in previous experiments. However, the fact that very high concentrations of activity were de-

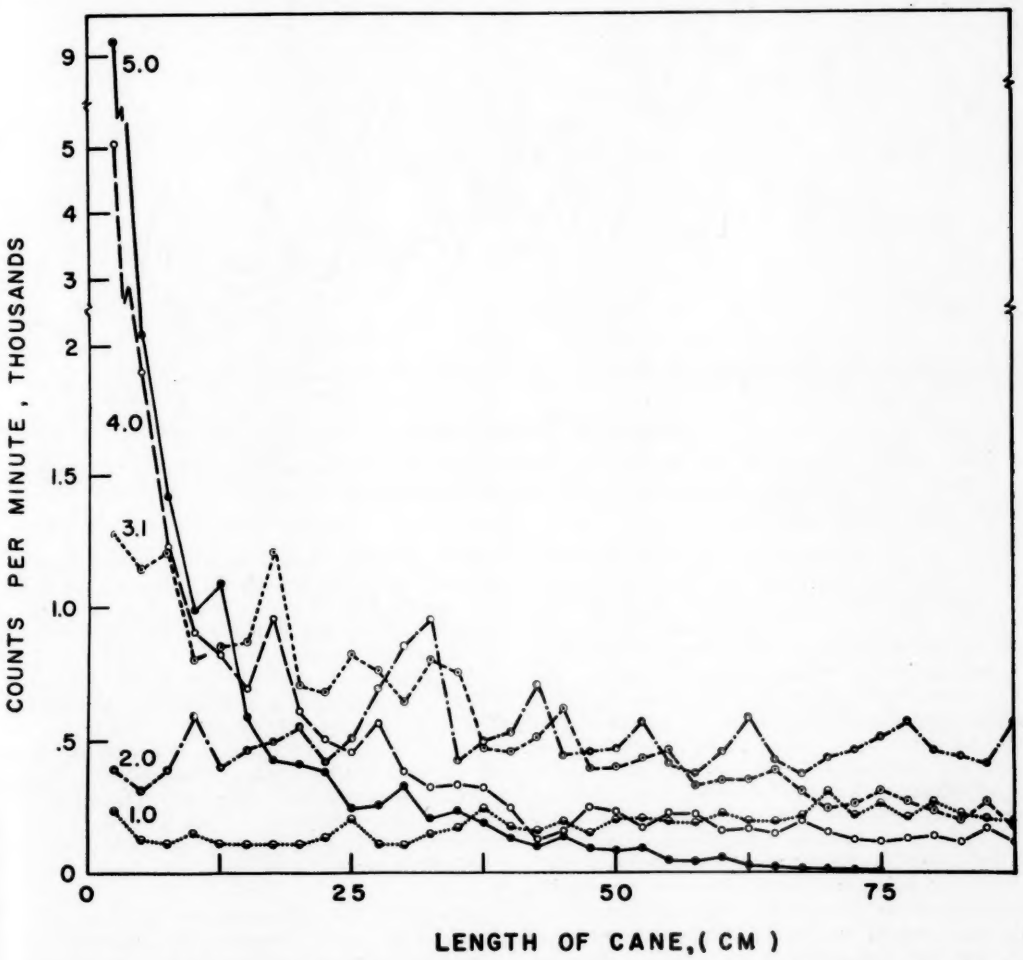

FIG. 2. Number of radiation cpm in 2.54-cm sections of grape canes showing the distribution of Zn^{65} along the cane. Each cane was flushed with a solution of medium activity Zn^{65} adjusted to one of the following pH values: 1.0, 2.0, 3.1, 4.0, and 5.0.

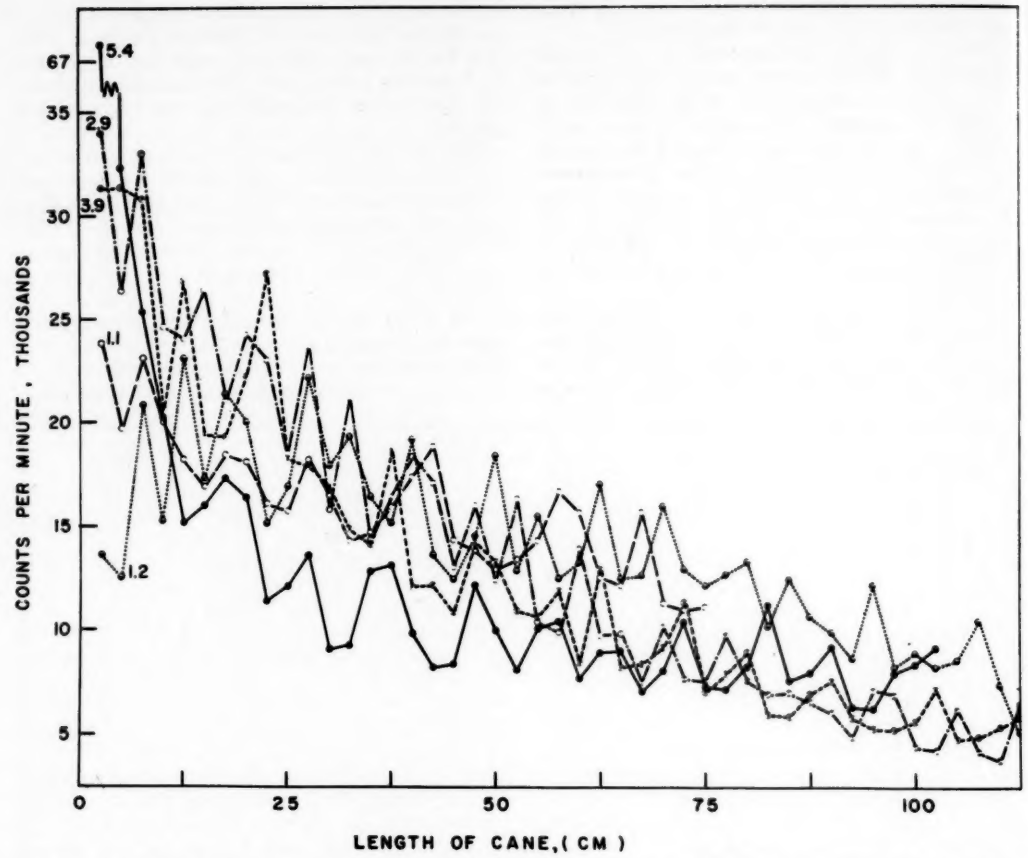

FIG. 3. Number of radiation cpm in 2.54-cm sections of grape canes showing the distribution of Zn^{65} along the cane. Each cane was flushed with a solution of high specific activity $Zn^{65}SO_4$ adjusted to one of the following pH values: 1.1, 1.2, 2.9, 3.9, and 5.4.

posited from the solution of pH 4.5 within the first 5 cm of cane, much more than was deposited in a region from any of the other solutions, shows that the canes had the capacity to adsorb more radioactive zinc, at least at this higher pH. After the first 10 cm of cane the deposit of activity from the pH 4.5 solution dropped below that of the other solutions. More radioactivity was deposited in the first few cm of cane from solutions of pH 3.9 and 2.9 than from solutions of pH 1.1 and 1.2. The rate of adsorption of radioactive zinc along the canes as shown by the slope of the lines after the first 10 to 15 cm was fairly uniform and continued at this rate through the 1.5-m length of the canes. This decline might indicate that the pH of the solutions passing through the canes was altered. The slope of the graphs after about 10 cm could be due to a decrease in flushing solution concentration but may also represent a decrease in cane diameter and a corresponding decrease in water-conducting vessels.

ADSORPTION: If the deposit of zinc salts along the cane is adsorption, then it should be possible to saturate the adsorption area in the cane and create an even distribution of radioactivity along its length, even at the high pH values, with solutions of high $ZnSO_4$ concentrations.

Flushing solutions were made using $ZnSO_4$ and $Zn^{65}SO_4$. One ml of high level $Zn^{65}SO_4$ was added to 9 ml of 25 % zinc sulfate ($ZnSO_4 \cdot 7\,H_2O$) in distilled water. The 10-ml lots of solutions were adjusted to pH values 1.5, 2.0, 3.5, and 5.4 and flushed through grape canes as previously described. The canes were allowed to stand overnight. They were then cut into pieces 2.54 cm long and the radioactivity of each determined as before. The results are shown in figure 4, A and B.

Except for the adsorption from the solution adjusted at pH 2.0 the activity in this experiment was fairly evenly distributed along the canes indicating a uniform deposit of zinc salt. Why the deposit of zinc salts was greater from the pH 2.0 solution is not understood. In earlier experiments, such deposits at the

introducing end of the cane were noted from solutions of pH values of 2.3 to 3.0. But just why the curve should swing up and then down is not evident.

To test this technique of determining radioactivity in the cane sections these same cane sections were again counted nearly a year later and the counts corrected for decay time of Zn^{65}, and for coincidence losses as before. These second counts made a year later are shown in figure 4, A and B as unfilled circles. With but few exceptions they duplicated the first ones very closely and show that the counting method was satisfactory.

In charting the distribution of radioactivity in the 2.54-cm sections of the canes, especially as shown in figures 3 and 4, there was a lot of up and down fluctuation in the general trend of the curves. After matching the cane pieces together along the graph it was evident that the high peaks in the distribution curve

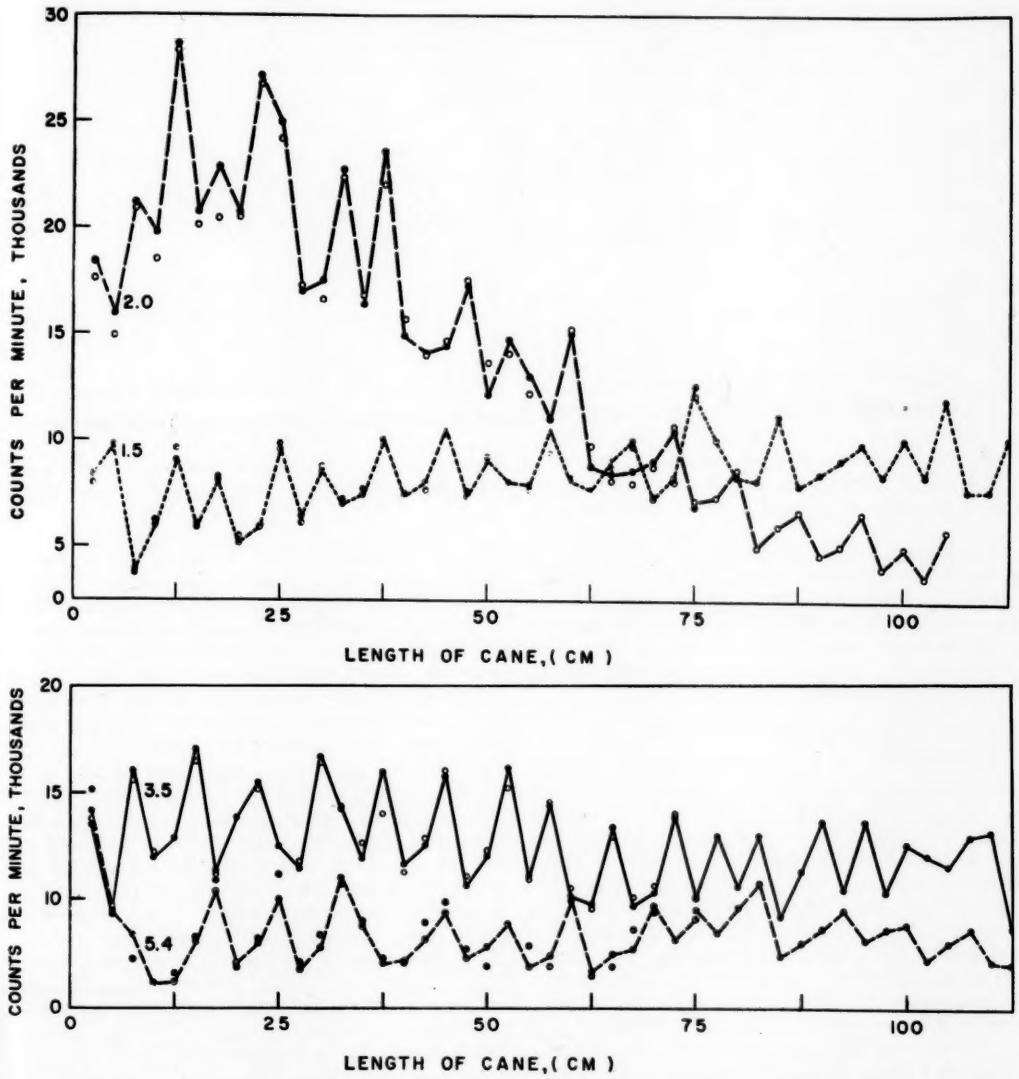

FIG. 4. Number of radiation cpm in 2.54-cm sections of grape canes showing the distribution of Zn^{65} after each was flushed with a solution of high activity $Zn^{65}SO_4$ which was made up with a 25% non-radioactive zinc sulfate ($ZnSO_4 \cdot 7\,H_2O$) solution. A) solutions adjusted to pH values 1.5 and 2.0; B) solutions adjusted to pH values 3.5 and 5.4. The filled circles indicate the points of the original readings in 1952 and the open circles are the readings made one year later on the same pieces of grape canes and corrected for decay time.

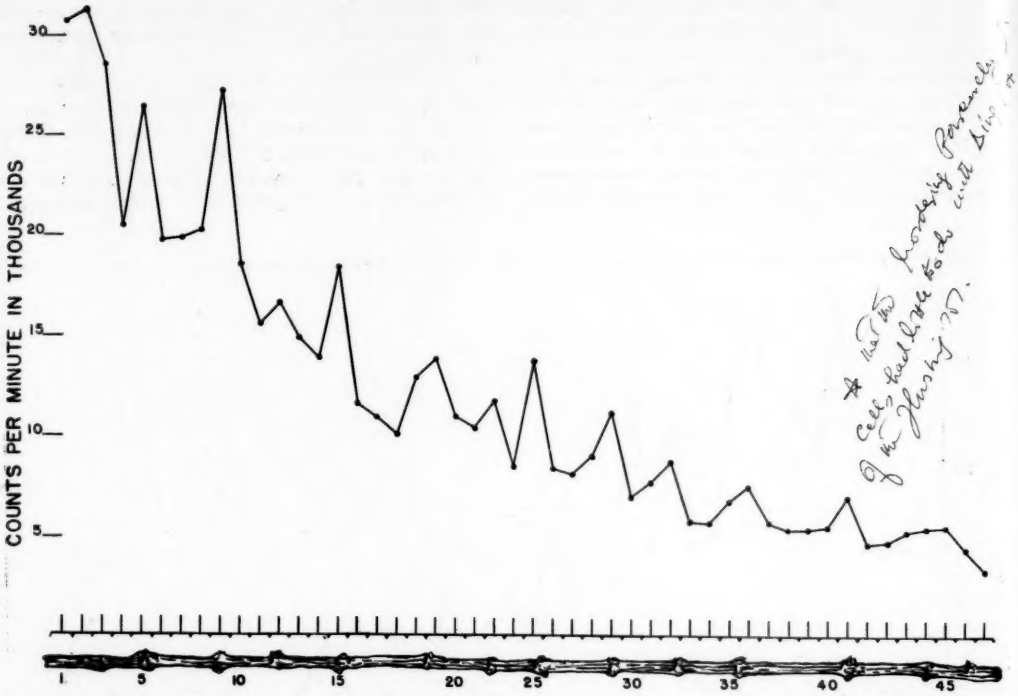

FIG. 5. Number of radiation cpm in 2.54-cm sections of grape cane showing the distribution of Zn^{65} in it after flushing with a $Zn^{65}SO_4$ solution. The sketch of the cane shows the relation of the high points in activity to the nodes and suggests accumulation in the leaf traces.

were associated with the nodes. This is illustrated in figure 5 where a cane has been drawn out to show the position of each node in relation to the activity shown in each section of the cane. The build-up in activity at each node was probably due to solution being side-tracked into the vascular leaf traces that terminate at the nodes in the dormant canes.

SUMMARY AND DISCUSSION

Results of flushing Thompson Seedless grape canes with different radioactive $Zn^{65}SO_4$ solutions show that the Zn^{65} was fixed or more likely adsorbed on the vessel walls; that flushing the same cane with distilled water dislodged very little of the Zn^{65}, but that flushing with a solution of non-radioactive $ZnSO_4$ caused a marked displacement of the Zn^{65} from the vessels in the cane. These studies further show that Zn^{65} was adsorbed rapidly from solutions at high pH values, that as the pH of the flushing solutions was decreased the adsorption of Zn^{65} also decreased per unit of length but increased over a longer total length especially in the first 30 to 60 cm of cane, and that the pH of the flushing solution was changed to a higher pH within the canes.

When the canes were flushed with a solution of Zn^{65} having activities so high that the exudate contained measurable radioactivity, the pattern of deposition along the cane was similar to that of the weaker solutions although actual values were, of course, higher. Also, the pattern did not change very much at the different pH values. However, when the flushing solution containing the same specific activity of radioactive zinc was made up with a 25 % solution of non-radioactive zinc sulfate solution, the cane became saturated with the zinc sulfate. Under these conditions the distribution of radioactivity was fairly uniform throughout the cane and did not change with the pH of the solution except at about pH 2.0.

The decrease in adsorption of Zn^{65} salts along the cane in a pattern as illustrated in figures 1 to 3 may have been due to the decrease in the number of vessels as they were side-tracked into nodes and leaf traces progressively from base to tip of cane. This is supported by the high peaks of radioactivity at the nodal points (fig 5). Contrary to this, however, the flushing solutions of high zinc sulfate concentrations deposited radioactivity fairly uniformly along the cane.

These results indicate that the $Zn^{65}SO_4$ is probably adsorbed to the vessel walls since it was not freed with distilled water but was displaced by $ZnSO_4$. The deposit was in a pattern that might be expected from passing a solution of salts over an adsorption surface.

The cells bordering the vessels apparently contributed to the change in pH of the flushing solutions as they moved through the cane. It is also probable that H ions may have been adsorbed from the solution and

that the bordering parenchyma cells had little to do with changing the pH of the flushing solutions. The nature of the materials or processes which brought about this change was not investigated.

Much of the preliminary work in these studies was done in cooperation with the late W. O. Williams, Department of Viticulture, University of California, Davis.

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VERNALIZATION IN PEAS[1]

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A great number of plants have been shown to require a cold treatment for the subsequent formation of flowers or for the hastening of flowering (6, 10). A plant in which flowering behavior is influenced by cold treatment applied in the seedling stage is said to be vernalizable.

McKee (5) has shown that a cold treatment during germination will hasten flowering in peas. Despite this evidence, peas have not generally been recognized as being naturally vernalizable. Peas have, however, been used extensively as experimental material in studies of the effect of various chemicals and growth substances on flowering. Leopold and Guernsey (7) found that the application to Alaska pea seeds of low concentrations of auxin (NAA) combined with a short cold treatment is effective in decreasing the number of nodes to the first flowers. Others, notably Fries (1) and Haupt (3, 4), have shown that the application to seeds or to seedlings of substances other than auxins can likewise affect the flowering of peas in terms of time to flower or in terms of the number of nodes to the first flower.

It will be shown in this paper that at least two varieties of peas, both late varieties, are naturally vernalizable, in the sense that their subsequent flowering behavior is influenced by a cold treatment given

[1] Revised manuscript received June 5, 1956.
[2] Report of work supported by a grant from the National Science Foundation.

Table I

The Effect of a Cold Treatment (Two Experiments) on the First Node to Flower in Two Varieties of Pea (Pisum sativum L.)

Days of cold treatment	Varieties of pea			
	Unica		Zelka	
	Vernalized	Devernalized	Vernalized	Devernalized
	Temp 7° C			
0	18.6 ± 0.5	.....	18.0 ± 0.61	.....
5	18.4 ± 0.52	19.2 ± 0.46	17.0 ± 0.74 *	18.6 ± 0.86
10	17.3 ± 0.71 *	19.0 ± 0.75	16.5 ± 0.97 *	17.5 ± 0.92 *
20	16.2 ± 0.82 *	18.5 ± 0.53	15.0 ± 0.71 *	16.8 ± 0.45 *
30	16.0 ± 1.11 *	19.1 ± 0.9	14.5 ± 0.75 *	17.0 ± 0.75 *
	Temp 4° C			
0	18.63 ± 0.52 **	19.5 ± 1.0	18.2 ± 0.42 **	18.17 ± 0.85
5	17.7 ± 0.47 *	18.57 ± 0.79	18.4 ± 0.55	19.2 ± 0.73
15	16.8 ± 0.41 *	18.0 ± 0.82	16.57 ± 0.79 *	17.25 ± 0.5
25	16.0 ± 0.57 *	17.13 ± 0.53 *	15.3 ± 0.68 *	16.8 ± 0.45 *

Values given are the means (± the standard error) of a minimum of 9 plants per treatment.
* Difference from controls significant at the 1 % level.
** No significant difference between vernalized and devernalized controls.

during the seedling stage. It will also be shown that the cold treatment has a distinct and separable effect on the vegetative development of the plant as well as on flowering, and that the pea is vernalizable only over a rather limited range in the physiological development of the plant. These facts make this plant an experimentally interesting one.

The effect of a cold treatment on the vegetative development of plants, has been shown, for example, in Petkus winter rye (8) where vernalization results in decreased formation of tillers. However, it has not been shown whether the vegetative effect is independent of flowering or whether it is a secondary reaction dependent on the effect of cold treatment on flowering.

Materials and Methods

Two pea varieties, Unica and Zelka (commercial varieties obtained from Holland) were used in these experiments. Both are late varieties which when grown under optimal greenhouse growing conditions of a long day (16 hrs) and relatively cool temperatures (17 to 20° C) will flower after the 18th node.

Seeds were soaked in tap water for from 4 to 6 hours at 20° C and planted in containers filled with a mixture of 50 % vermiculite and 50 % crushed rock. The containers were then put into constant temperature rooms at either 4 or 7° C in the dark for periods up to 30 days and watered when necessary with Hoagland's nutrient solution.

Series of 30 plants were removed at intervals. In each series half of the plants were put directly into the normal growing conditions, which in these experiments consisted of 8 hours of natural light at 20° C, 8 hours of artificial light at 17° C and 8 hours of darkness at 17° C. The remaining plants of a series were maintained under devernalizing conditions of 30° C (16 hours of artificial light) for 10 days. At the end of the 10-day devernalizing treatment the plants were put into the normal growing conditions described above.

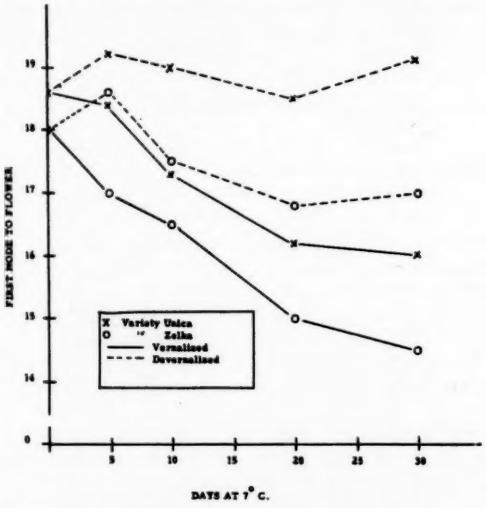

Fig. 1. The effect of a cold treatment on the first node to flower. The broken curves show the effect of a high temperature treatment of 30° C for 10 days immediately following the cold treatment.

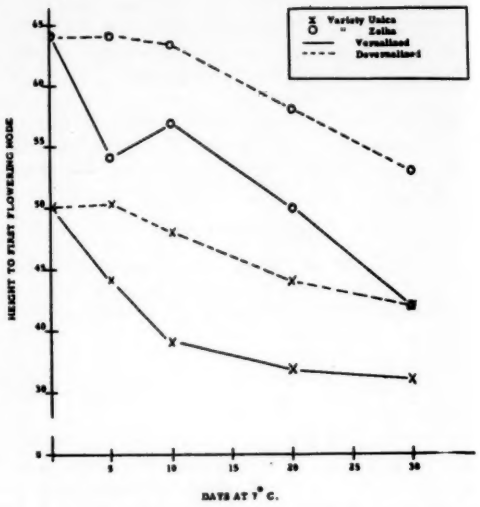

Fig. 2. The effect of a cold treatment on the height (in mm) to the first flowering node. The broken curves show the effect of a high-temperature treatment of 30° C for 10 days immediately following the cold treatment.

Measurements of flowering behavior were made by counting the number of nodes formed before the first flower appeared. Measurements of the vegetative development of the plants were made by measuring the height from the first bract to the first flowering node. Data were recorded after the internode below the flowering node had completely elongated.

Experimental Results

The results of two of a series of experiments showing the effect of a cold treatment on the first node to flower are presented in table I and figure 1. Replication in each of these experiments was from 9 to 15 plants per treatment. Variability is expressed as the standard error of the mean. The two series shown are representative of the results obtained in other similar experiments.

For both varieties cold treatments of 5 days or more decreased the number of nodes to the first flower as compared with the control. The differences are statistically significant at the 1 % level. It can also be seen from this table that the devernalizing treatment significantly delayed flowering as compared to the control. While in the variety Unica, the heat treatment which followed the vernalizing treatment completely reversed the effect of cold; in the variety Zelka complete reversal did not occur although the heat treatment following a cold treatment did significantly delay flowering.

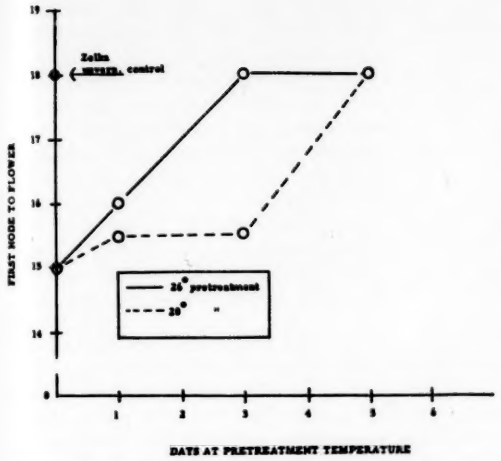

FIG. 3. The effect on the first node to flower of a pretreatment at 20 or 26° C prior to a cold treatment at 7° C for 25 days.

Height to the first flowering node may be used as another measure of vernalization. That there is an inductive effect of a cold treatment on the height to the first flowering node can be seen from figure 2. In both varieties devernalization results in some increase in the height to the first flowering node but high temperature treatment following vernalization is apparently not as effective on vegetative development as it is on flowering. This is especially true when we consider the variety Unica in which the high temperature treatment completely reverses vernalization with respect to flowering.

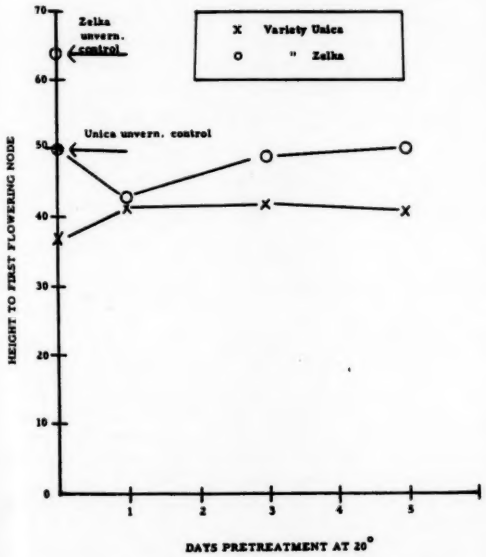

FIG. 4. The effect on the height to the first flowering node of a pretreatment at 20 or 26° C prior to a cold treatment at 7° C for 25 days.

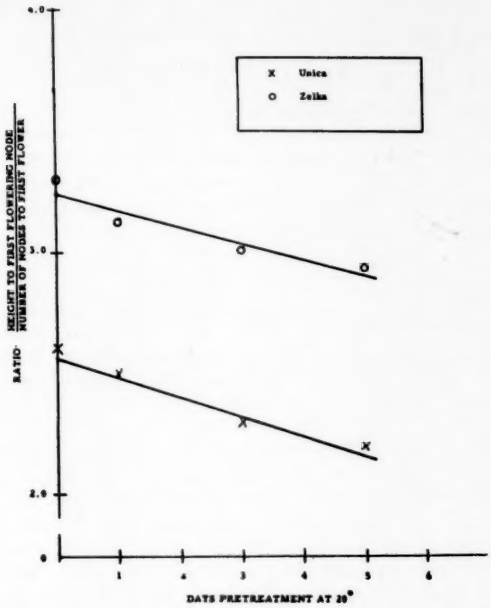

FIG. 5. Average internode length as a function of a pretreatment at 20° C.

In a second series of experiments plants were germinated at a high temperature prior to cold treatment in order to determine whether there was a critical time in the development of the pea seedling during which it was most receptive to a cold treatment. These plants were germinated at 20 or 26° C for periods up to 5 days. At the end of this time the seedlings were given a cold treatment at 4 or 7° C for 25 to 30 days; a period of cold sufficient to result in a maximum effect of the cold.

Figure 3 shows the results of one such experiment in which seeds of the variety Zelka were used. It is apparent that the longer the seeds are germinated at 20 or 26° C, the less effective is the following cold treatment in reducing the number of nodes to the first flower. With a 20° C pretreatment the critical period lies somewhere between 3 to 5 days since after three days at 20° C the plants can no longer be vernalized. When the plants are germinated at 26° C they remain vernalizable for 1 to 2 days. Three or more days of pretreatment at 26° C prevents subsequent cold treatment from exerting an effect on flowering.

The initial germinating temperature has no apparent effect on the height to the first flower. Figure 4 shows the results of a high temperature pretreatment on height to flower. These are the same plants from which the flowering data of figure 3 were taken. The decrease in height of the vernalized plants as compared to the unvernalized plants remains the same throughout the range of the pretreatment periods.

If the data of figure 4 are plotted as in figure 5, i.e., ratio of height to the first flowering node to the node at which the first flower appears, then it can be seen more clearly that flowering and vegetative development are independently affected by vernalization. If the decrease in height was due only to the decrease in the number of nodes to the first flower, the slopes of the lines should be zero when actually they are less than zero. The figures on this graph can also be read as the average node length.

Discussion

The results of these experiments show that peas, at least the varieties used in these experiments, can be vernalized and that the inductive effect of cold treatment can be reversed by high temperature treatment (i.e., they can be devernalized). It is interesting to note that in the 1948 report from the Netherlands of the Rijksinstituut voor Rassenonderzoek van Landbouwgewassen (9) on field tests of pea varieties, both varieties used in these experiments (Zelka and Unica) are considered to be relatively early varieties, flowering at the 12.9 and 14.5 nodes respectively. Since peas are normally planted early in spring when the temperatures are sufficiently low to allow the vernalization process to be consummated, it seems likely that vernalization has in fact actually occurred in the reported field trials.

In addition to the fact that the pea varieties can be vernalized and devernalized two other facts of interest have emerged:

1) There appears to be a distinct and separable effect of cold treatment on the vegetative development of peas, an effect which, like the effect of flowering, is inductive. Devernalization only partially reverses the effect of vernalization on vegetative development in the variety (Unica) in which devernalization completely reverses the vernalization with respect to flowering. In the variety Zelka this is not as clear, although the degree of reversal due to high temperature is far less for vegetative development than it is for flowering. Both the processes effected by a cold treatment, while obviously going on simultaneously must be distinct. This is especially apparent in the experiments in which the germinating seeds are pretreated at 20 or 26° C prior to receiving the vernalizing treatment. The fact that pretreatment of the germinating seed for a short period (3 to 5 days) prior to a cold treatment makes the plant insensitive to cold with respect to flowering, although it remains sensitive to a cold treatment with respect to the vegetative development, suggests that the effect of a cold treatment can be separated into a) a vegetative effect, and b) a reproductive effect, both of which are inductive. The former could be termed *vegetative vernalization* as opposed to *vernalization* which usually refers to the inductive effect of a cold treatment on flowering alone.

2) The high temperature pretreatment experiments indicate that there are physiological limits during which flowering can be effected by a cold treatment. With a pretreatment at 20° C of germinating seeds for a period up to 3 days, vernalization is still possible. With a pretreatment at 26° C for one day vernalization is still possible. But, with pretreatments of more than 3 days at 20° C and more than one day at 26° C vernalization is no longer possible. This is significant when one considers that dissection of growing points of samples of plants pretreated at 20° C up to 5 days, reveals no difference in the number of nodes from the number present in the dry seeds. At 26° C there is no change in the number of nodes present up to the fourth day; only elongation of the epicotyl occurs during these periods at both temperatures.

Since no new nodes have been laid down during the critical period of the pretreatments, some physiological changes rather than morphological changes must take place during this time. The plants appear to have passed through a critical stage in their physiological development such that they can no longer be vernalized.

These experiments seem to delimit the period in development which is most critical in studies of vernalization and indicate the developmental stages which should be used in studies of the biochemical changes occurring during the vernalization of peas.

Summary

The inductive effect of a cold treatment on flowering in peas has been studied. It has been found that the two late varieties studied are normally vernalizable with respect to both flower formation and vegetative development and that these two phenomena can be separated.

A pretreatment during germination at 20 or 26° C for up to 5 days prior to the optimal cold treatment results in a progressive loss of ability to be vernalized. Plants remain vernalizable for up to 3 days at 20° C. At 26° C they remain vernalizable for only 1 or 2 days.

The pretreatment at either temperature has no effect on vernalization with respect to vegetative development.

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EFFECT OF SULFHYDRYL INHIBITORS ON RUBIDIUM ABSORPTION BY EXCISED MUNG BEAN ROOTS[1]

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In recent years several investigators have used metabolic inhibitors in their studies on salt absorption by plants. Ordin and Jacobson (9) have reviewed publications in this field. All of these workers have found that at appropriate concentrations the inhibitors reduced salt uptake. This paper reports unique effects of several sulfhydryl[2] inhibitors on the absorption of Rb by excised mung bean roots (*Phaseolus aureus*). Under certain conditions some -SH agents have been found to increase greatly the uptake of Rb. Other conditions have resulted in these inhibitors drastically decreasing the absorption of Rb.

Material and Methods

Mung beans were grown at 25° C in contact with an aerated 10^{-4} M $CaCl_2$ solution which was changed daily. Root tips were excised in 1-cm lengths from three-day-old seedlings. Fifty root tips were placed in 50 ml solution of 10^{-4} M RbCl containing tracer amounts of Rb^{86} (less than 5 μc/l) and various concentrations of a -SH inhibitor. The inhibitors used were phenylmercuric nitrate, $CuSO_4$, iodoacetic acid, and 3-amino-4-hydroxy-phenyldichloroarsine hydrochloride.[2] In one series of experiments the absorption medium contained 10^{-3} M $Ca(NO_3)_2$. The solution was vigorously aerated during the absorption period of 30 min at 25° C. After absorption, the radioactive solution was rapidly siphoned off and the roots washed several times with 10^{-2} M inactive salt solution and water. The root segments were then placed in an aluminum dish, dried at 100° C, and the radioactivity assayed in the conventional manner.

Experimental Results

Typical results of many experiments showing the effects of the -SH inhibitors on Rb absorption by mung bean roots in the absence of Ca are presented in figure 1. The strong inhibitors such as Cu and PMN brought about an unexpected and surprisingly large increase in Rb uptake at low concentrations (less than 2 ppm). At higher concentrations Cu tended to depress Rb uptake. High concentrations of PMN were not used because of its insolubility in water. On the other hand, the weaker -SH agent, AHP, brought about much smaller increases in Rb absorption; while the weak inhibitor, IDA, decreased Rb uptake as previously reported by others (9).

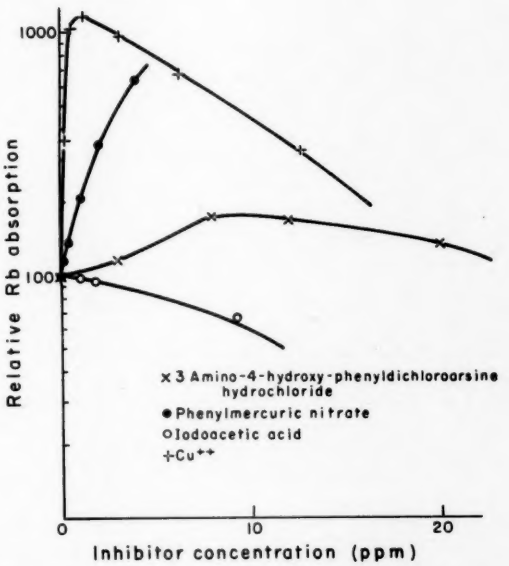

Fig. 1. Effect of sulfhydryl inhibitors on the absorption of Rb by excised mung bean roots in the absence of Ca.

The presence of 10^{-3} M Ca in the absorption medium drastically changed the direction of the effects of the strong -SH inhibitors on Rb uptake (fig 2). Instead of increasing Rb absorption, Cu and PMN at very low concentrations (less than 1 ppm) greatly reduced Rb uptake in the presence of Ca. In

[1] Received June 12, 1956.

[2] Abbreviations used: -SH, sulfhydryl; PMN, phenylmercuric nitrate; IDA, iodoacetic acid; AHP, 3-amino-4-hydroxy-phenyldichloroarsine hydrochloride; RNA, ribonucleic acid; RNase, ribonuclease.

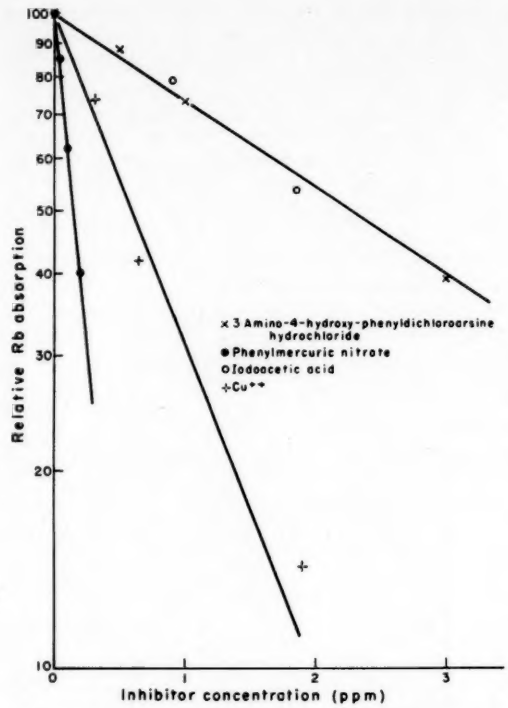

FIG. 2. Interaction of sulfhydryl inhibitors and Ca on the absorption of Rb by excised mung bean roots. The absorption medium contained 10^{-4} M RbCl, 10^{-3} M $Ca(NO_3)_2$, and various concentrations of inhibitors except in the case of the Cu treatment. The Cu-treated roots were pretreated with various concentrations of $CuSO_4$ for one min and washed before placement in the absorption medium containing RbCl and $Ca(NO_3)_2$.

the case of Cu this depressing effect was observed only when the roots were first pretreated with $CuSO_4$ for one min before placement in the RbCl solution containing $Ca(NO_3)_2$. When Ca and Cu were added together in the absorption medium, the Ca prevented any effect of the Cu. On the other hand, no such antagonism was observed between Ca and PMN when they were added together. The weaker -SH agents showed little interaction with Ca and were effective only at fairly high concentrations. When a monovalent cation such as Na was used in the form of $NaNO_3$ in place of Ca, no interaction was observed between the Na and the strong -SH inhibitors as between Ca and the strong -SH inhibitors. Cysteine and glutathione were able to reverse completely the Cu effect and partially the PMN effect.

The interaction between the effects brought about by strong -SH poisons and by Ca on Rb absorption appears to take place at low concentrations of Ca. Table I shows the effect of PMN and increasing Ca concentration on Rb absorption by excised mung bean roots. It can be seen from the data that even low concentrations of Ca have a marked depressing effect on the "enhanced" Rb uptake brought about by PMN. The data also indicate that the Ca reduction of the PMN effect increases with increasing Ca concentration.

DISCUSSION

From the results reported in this paper, it is obvious that certain -SH groups play an important part in the normal absorption of ions by mung bean roots. What that role is can not be unequivocally answered in these in vivo experiments. However, some of the inhibitory effects of the weak -SH agents could be due to their poisoning of some critical metabolic reactions which are necessary for normal salt absorption. But, such an explanation does not appear to explain adequately the unusual effects of the strong -SH inhibitors, which can greatly increase or decrease Rb absorption depending upon the presence of Ca in the absorption medium. One probable explanation of the striking effects of PMN and Cu on increasing Rb uptake is that these poisons caused changes in the permeability of the membrane which limits the active uptake of ions. This point was studied by treating excised roots, which had previously absorbed Rb^{86}, with $CuSO_4$ for short periods. After washing, the Cu-treated and control roots were then placed in 10^{-4} M inactive RbCl. The loss of Rb^{86} from the roots was then measured after suitable intervals. Both control and Cu-treated roots showed little or no loss of Rb^{86} after 15 and 30 min in the inactive RbCl. Obviously, if permeability changes occurred in the cells following the Cu treatment, the loss of Rb^{86} from the treated roots would be very much higher than untreated roots unless the unlikely possibility occurred that only permeability into the cells was changed. In addition, a few experiments with P^{32} showed that PMN had only a moderate inhibiting effect on phosphate absorption. This inhibition increased with increasing PMN concentration with Ca having little or no effect on the course of the inhibition. Lewin (7) likewise has found that -SH poisons inhibited silicon uptake by diatoms. A somewhat comparable phenomenon has been observed on the effect of traces of Cu on the absorption of glycerol by human erythrocytes. The literature on this subject has been reviewed by Le Fevre (6) and Jacobs (4).

TABLE I

EFFECT OF PHENYLMERCURIC NITRATE AND INCREASING CALCIUM CONCENTRATION ON RUBIDIUM ABSORPTION BY EXCISED MUNG BEAN ROOTS

COMPOSITION OF ABSORPTION MEDIUM		Rb ABSORPTION (CPS)
PMN (PPM)	Ca^{++} (M)	
0	0	10.3
2.0	0	19.8
2.0	10^{-5}	9.9
2.0	5×10^{-5}	5.1
2.0	10^{-4}	4.2
2.0	10^{-3}	3.2

These workers and coworkers have found that trace amounts of Cu inhibited uptake of glycerol by erythrocytes without affecting the uptake of some other compounds. They believe the Cu effect to be on -SH groups at cell surfaces. Unless permeability changes in the cells can be restricted only to cations, the strong -SH inhibitors probably affect cation absorption through some mechanism other than permeability changes. In addition, the drastic effects of Ca on PMN- and Cu-treated roots are also difficult to ascribe to permeability changes. Ca, as found by Viets (16) and others (11, 14), stimulates salt uptake in untreated roots.

If the results reported here can not be attributed to permeability changes, perhaps an explanation can be found in one of the current hypotheses on ion absorption. The more popular hypotheses do not offer much hope that an adequate explanation can be found in any one of them. They have been recently reviewed by Overstreet and Jacobson (10), Lundegårdh (8), and by Steward and Millar (13). However, a suitable explanation can be found in the less well-known postulate made recently by Lansing and Rosenthal (5) in which RNA[2] plays the role of an ion-binding carrier compound in salt absorption. Incidentally, Steward and Millar (13) have suggested that RNA takes part in salt absorption through its role in protein synthesis. Lansing and Rosenthal have found that treating Elodea cells with RNase[2] brought about a reduction in Ca uptake. From these results they suggested the role of RNA as an ion-binding carrier compound in salt absorption. They have cited several biochemical and cytological data which make their proposal plausible. Tanada (15), using excised mung bean roots, has recently confirmed their findings that RNase has a marked effect on salt uptake. In using Lansing and Rosenthal's postulate to explain ultraviolet and Ca effects on ion uptake by excised mung bean roots, Tanada (14) has slightly modified their suggestion by identifying the carrier as a ribonucleoprotein with the negatively charged nucleic acid binding cations and the positively-charged protein binding anions.

Now, if the ribonucleoprotein hypothesis is used to explain the results reported here, one can visualize the nucleic acid and the protein moieties being held together by certain critical H bonds formed between -SH groups of the protein and other H-bonding groups of the nucleic acid. Henry and Stacey (2) have found that -SH groups in the cytoskeleton have to be reduced before RNA becomes attached to it. Immobilization of the -SH groups by strong -SH agents would break the H bonds. Without these critical H bonds with the -SH groups of the protein, the nucleic acid would be held weakly or become separated from the protein. Under this condition changes in the spatial configuration of the RNA could result and make available more sites for cation binding. This could explain the increased uptake of Rb by the action of the strong -SH agents, Cu and PMN, in the absence of Ca. It is likely, however, that the protein determines to a large extent the properties of the RNA, and the RNA is physiologically stable only when combined with the protein (3). If "free" RNA were unstable, small amounts of Ca could have marked deleterious effects on the physico-chemical properties of the "free" RNA and result in the RNA losing much of its cation-binding potential. Ambrose and Butler (1) and Shack et al (12) have found that small concentrations of polyvalent cations have large effects on certain physico-chemical properties of nucleic acids and nucleoproteins.

At present, until more information is obtained on salt absorption, it would be premature to speculate on the actual mechanics of ion absorption involving a ribonucleoprotein as an ion-binding carrier compound. However, if the above explanations present the true picture on the action of -SH agents, the effect of ultraviolet irradiation on Rb absorption reported recently by Tanada (14) would be suitably explained. Tanada found that ultraviolet irradiation has an effect on Rb absorption by mung bean roots similar to that brought about by the strong -SH inhibitors. Ultraviolet irradiation increased Rb uptake in the absence of Ca; but in the presence of Ca, it decreased Rb absorption. These results indicate that one of the major results of ultraviolet irradiation on salt absorption could be the oxidation of -SH groups of a ribonucleoprotein.

Summary

The absorption of Rb by excised mung bean roots has been found to be markedly affected by strong -SH inhibitors. Cu and phenylmercuric nitrate at low concentrations have been found to increase markedly Rb uptake in the absence of Ca. Rb absorption in the presence of Ca, however, was greatly reduced by these inhibitors. Weaker -SH poisons showed little or none of these effects. The suggestion was made that Rb absorption is mediated by a ribonucleic acid and protein complex which is held together by H bonds formed by certain critical -SH groups on the protein moiety, and that the strong -SH agents bring about their effects by their action on these -SH groups.

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BRIEF PAPERS

IDENTIFICATION OF PHOSPHORYL CHOLINE AS AN IMPORTANT CONSTITUENT OF PLANT SAPS[1,2]

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Several phosphorylated compounds have been reported in xylem saps of a variety of plants by Tolbert and Wiebe (8). They characterized the major compound by its R_f value (0.9 in phenol: 0.3 in butanol–propionic acid–water), apparent non-ionic character, acid stability and phosphatase hydrolysis to orthophosphate.

In studying phospholipid intermediates of plants we have found that phosphoryl choline is the major phosphate ester in plant saps. This compound has been identified in animal and bacterial phospholipid metabolism but has not been reported as a constituent of plant saps (1).

The major part of the labeled phosphoryl choline ($P^{32}C$) which occurs in barley leaves was extracted with 80 % ethanol and separated by two dimensional paper chromatography (4). The compound was detected by virtue of the radioactivity incorporated during growth of the seedling plants in P^{32} nutrient or with C^{14} incorporated by several days photosynthesis in $C^{14}O_2$. The very slow carbon labeling with $C^{14}O_2$ may be contrasted to the more rapid synthesis of PC^{14} with labeled choline precursors. PC is identical with a labeled product of $HC^{14}OOH$ and $H_2C^{14}O$ feeding experiments. The unknown compound U_1 isolated from barley fed $HC^{14}OOH$ by Tolbert (9) is PC. Glycolic-2-C^{14} is partially converted to PC when vacuum infiltrated into barley leaves.

Barley seedlings were placed in small vials of tap water containing 0.2 mc radiophosphate (pH 6). After 20 hours at 200 ft-c the leaves were cut and extracted with boiling 80 % ethanol. Plants to be labeled with C^{14} and P^{32} were placed in a test tube containing dilute $KH_2P^{32}O_4$ (0.2 mc) and $C^{14}O_2$ (0.1 mc). After five days (200 ft-c) the leaves were extracted. The concentrated extract from one gm of tissue was chromatographed on a sheet of Whatman No. 4 paper. A compound with R_f corresponding to that reported by Tolbert and Wiebe was identified by cochromatography with authentic $P^{32}C$. The exudate from the cut stem was then chromatographed and found in the same manner to contain orthophosphate and $P^{32}C$. Authentic PC and glyceryl phosphoryl choline, GPC, were kindly supplied by Professor D. J. Hanahan of the University of Washington.

The $P^{32}C$ was acid-stable. Its hydrolysis rate, determined from measurements of the freed radiophosphate isolated chromatographically, was similar to that reported for PC by Baer and McArthur (2). "Polidase S" (Schwarz Chemical Company) hydrolysis gave orthophosphate and choline-C^{14}. The authentic carrier choline was detected by Draggendorf's reagent (5) ($KBiI_4$) which gave a reddish-brown spot coincident with the C^{14} radioactivity.

The unknown compound was further characterized and identified as PC by electrochromatography with 0.1 M lactic acid (pH 2.5) as the electrolyte on filter paper strips (7). Neither PC nor the unknown compound migrated when on mixed or on separate origins, whereas orthophosphate migrated rapidly as an anion. When 0.1 M lactic acid adjusted to $pH < 1$ with hydrochloric acid was used as supporting electrolyte, PC and the unknown compound moved at slow, identical rates as cations while orthophosphoric acid migrated slowly as an anion. In 0.1 M potassium lactate adjusted to $pH > 11$ with KOH, PC and the unknown compound migrated slowly as an anion. Orthophosphate also migrated as an anion but much more rapidly than PC. All electrochromatographic studies (7) were run at a potential gradient of 7 volts per cm on strips of Whatman No. 4 filter paper 54 cm × 9 cm with origins at the middle. Nonradioactive compounds were detected using the spray reagent described previously (3) for detecting phosphorus. The $P^{32}C$ area was further resolved in methanol-ammonia-water solvent (60: 10: 30, v/v) (3) giving 10 to 15 % of a compound moving 18 % faster than PC and having the properties of glyceryl phosphoryl choline with which it chromatographed exactly.

The identification of phosphoryl choline in plant saps in amounts at least as high as 5 to 20 % (8) of the total phosphorus suggests its function as a phosphate carrier. The fact that it has now been found in phloem exudate after injection of radiophosphate into spinach and cucumber leaves indicates that the phosphorus transport by this means is not unidirectional. The phosphorylative mechanisms of plants occur in

[1] Received June 13, 1956.

[2] Authorized for publication on June 12, 1956, as paper No. 2070 in the journal series of the Pennsylvania Agricultural Experiment Station.

This research was supported in part by contracts No. AT(30-1)1876 and No. W-7405-Eng-26 with the Atomic Energy Commission.

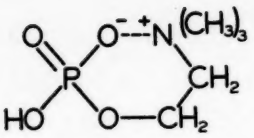

FIG. 1. Zwitter ion structure of phosphoryl choline.

phospholipid-containing structures, mitochondria and chloroplasts. The appearance of free PC and GPC suggests a relationship between phospholipid intermediates and possible mechanisms of phosphorylation. The transport of phosphorus as PC or GPC should be facilitated by the high organic-solubility, low adsorbtion and neutral character of these zwitter ions. Indeed, such a zwitter ion nature as indicated in figure 1 would lend itself to interpretation by the ion-carrier complex theory supported by Epstein (6). According to this theory ions combine with a carrier which enables them to traverse membranes of selective permeability after which they are released as free ions.

SUMMARY

The major phosphate ester in plant saps has been identified chromatographically as phosphoryl choline. It was readily labeled with P^{32} upon absorption of $KH_2P^{32}O_4$ through the roots and with C^{14} by vacuum infiltration of formate-C^{14}, formaldehyde-C^{14} and glycolate-2-C^{14}. However, C^{14} was incorporated very slowly by $C^{14}O_2$ photosynthesis. The zwitter ion structure and organic solubility of phosphoryl choline suggests that it may act as a phosphorus carrier capable of penetrating plant membranes.

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SOME PHENOTYPIC EFFECTS OF SINGLE GENE MUTATIONS ON RESPIRATORY CHARACTERISTICS OF MAIZE LEAF TISSUE[1,2]

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This report represents a preliminary survey comparing respiratory characteristics of mutant and normal corn plants from isogenic lines. Morphologically the same tissue was used for all comparisons and mutants and normal inbred seedlings were grown side by side under the same environmental conditions. Any differences which were detected presumably represented phenotypic expressions of single genes.

The genetic background of the corn plants employed in this survey was that of the University of Minnesota Agricultural Experiment Station Inbred, A 21. The mutant, dwarf-1 (1), was introduced into the inbred in 1940 and mutant progeny were backcrossed to the inbred five times. The mutant, brachytic-2 (3), was introduced in 1943 and homozygosity was similarly increased by backcrossing.

[1] Received July 12, 1956.

[2] Facilities for this research were provided by the Graduate School and by the Rockefeller Fund.

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Phenotypically the two mutants are morphologically dissimilar. Dwarf-1 is a seedling mutant which can be distinguished from normal siblings soon after germination. Leaves of the mutant are darker and broader. In stature the mature plant is a miniature of the normal attaining about one-third the height of the latter.

Brachytic-2 is indistinguishable from normal seedlings and can be identified only as the plants mature. Mutant plants attain about two-thirds the height of the normal.

These particular corn mutants have been the subject of anatomical and developmental studies in this department (2, 4, 6). The breeding program requisite to this research in developmental morphology provided the necessary seed from isogenic stocks and advantage was taken of the availability of this material to study differences in the corn mutants on a physiological rather than a morphological basis. In each experiment seed all from the same seed-lot was used to provide the experimental plants. In the case

of the one mutant, segregating seed from the same ear was used while the other mutant was employed in heterozygous condition using nonsegregating backcross progeny.

Leaf material for respiratory measurements was obtained from plants grown in sand in a constant temperature chamber. Plants were watered daily by subirrigation using alternately distilled water and either Knop's solution or Randolph and Cox's medium (5). Micronutrients were supplied in every fourth nutrient watering. Illumination was provided 14 hours per day by nineteen 100-watt incandescent bulbs 150 cm above the level of the sand. The air temperature was $26 \pm 2°$ C. Plants were numbered in rows and were sampled for measurement by means of a table of random numbers. All measurements were made on the third leaf which was removed at the sheath and sectioned transversely into one cm lengths before inserting into Warburg vessels.

Gas exchange measurements were made in a darkened constant temperature bath at 24° C by conventional manometric methods. The leaf tissue was floated on 0.01 M phosphate buffer pH 5.8 except in those experiments in which pH was a variable. The gas phase was air except for experiments under anaerobic conditions when helium was used. In each Warburg experiment all treatments were replicated and in most cases data used for comparisons were from the average of two vessels.

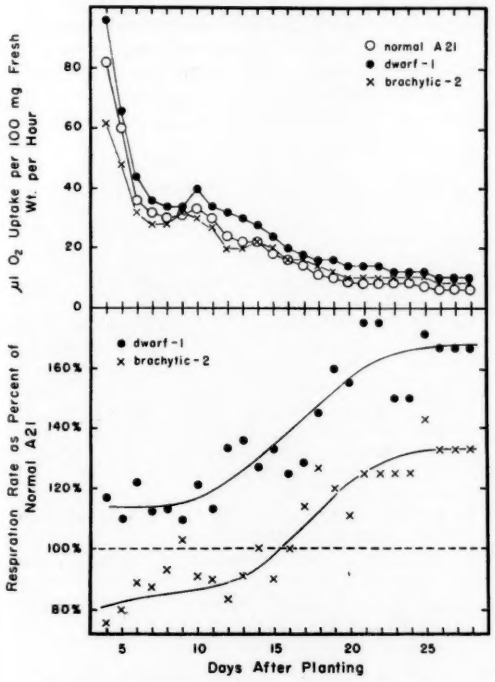

FIG. 1. Relation between leaf respiration rate and time after planting in 3 isogenic lines of corn. The genotype of each mutant differs from that of the normal (A 21) by a single gene. *Above.* Time course of oxygen consumption rate of third leaf tissue. *Below.* Same data with rates for mutant tissue plotted as percent of rate for normal.

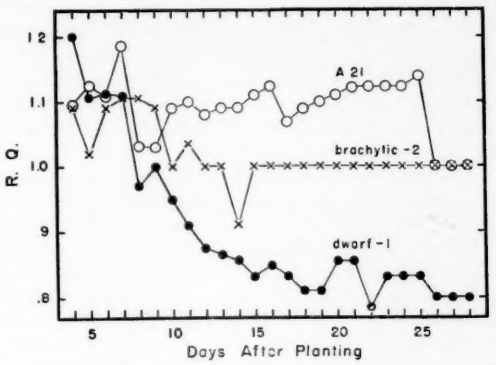

FIG. 2. Relation between RQ of third leaf tissue and time after planting in normal inbred corn (A 21) and in two mutants.

For the respiratory features which were investigated, both mutants were very similar to the inbred normal plants. Nevertheless, both quantitatively and qualitatively respiratory differences were observed between normal and mutant third leaf tissue. Significant differences in rates of oxygen uptake occurred throughout seedling growth as shown in figure 1. Such differences amounted to as much as 70 % and were reproducible not only with plants grown in the constant temperature chamber but also on greenhouse and on field grown populations. A somewhat similar but not identical set of time course patterns was found in rates of aerobic CO_2 production among the three lines. Except in seedlings less than 10 days old, each line had a significantly different RQ value as shown in figure 2.

Smaller but still significant quantitative differences were observed in the case of respiration stimulated by 0.10 M succinate where one mutant (dwarf-1) consistently exhibited slightly more succinate stimulation of oxygen consumption than did the normal throughout seedling development. (Average stimulation: 58 % in mutant, 51 % in normal). Likewise, in feeding experiments with 1.0 % glucose, one of the mutants (brachytic-2) responded consistently less than did the normal. (Average stimulation: 45 % in mutant, 71 % in normal).

In most respects, however, respiratory features of mutant and normal plants were similar. No significant differences were found between normal and either mutant when rates of anaerobic CO_2 production were compared. In all three lines substantially the same Meyerhof quotients were computed for plants of the same age. This quotient rose from nearly zero in 4-day-old plants to about 0.6 at 22 days. A Pasteur effect was discernible in all third leaf material after the tenth day.

At pH 5.8 the same sensitivity of oxygen consumption rate to sodium azide was found in each of the three lines. This applied to an azide concentration of 10^{-6} M (stimulatory) as well as 10^{-4} M (inhibitory). Azide had less effect on CO_2 production; thus in 10-day-old leaf tissue 5×10^{-5} M sodium azide increased the RQ from about unity to 1.65.

No significant differences were found in response to 2,4-dinitrophenol (DNP). Both mutants and the normal showed approximately the same stimulation of oxygen consumption rate (up to 160 % of controls) at low concentration (10^{-5} M) and inhibition at higher concentrations of DNP. Also the same pattern of dependence of DNP response upon pH was observed in each of the three lines. Carbon dioxide production showed even more pronounced DNP stimulation and inhibition. As was the case with azide, higher DNP concentrations caused more inhibition of oxygen reduction than of CO_2 production. The RQ of 15-day-old leaf material in the presence of 5×10^{-5} M DNP rose in all three lines to as high as 1.9.

That plants of three different lines with a high degree of homozygosity show some quantitative differences in respiratory rates is not in itself evidence that the differentiating genes modify directly the respiratory enzyme systems. Respiratory rate differences which become apparent when rates are calculated on a fresh weight basis may signify nothing more than a difference in plant moisture contents. Thus a difference may only reflect the basis upon which data are compared. If, instead of fresh weight, a comparison were made on a dry weight basis, or per mg nitrogen, or in some other way, these particular differences might vanish and possibly others arise. Some of the differences found might also result from as little as a 24-hour delay in germination of one line compared with another since rates were observed to change progressively and our only criterion of morphological age of tissue is chronological age. However, because one or both mutants differed from the normal with respect to some respiratory characteristics but not with respect to others, and because some of the differences which were detected changed during the course of seedling growth, we are led to suppose an influence of the mutant genes on the respiratory mechanism itself rather than to adopt a trivial explanation for those differences which we observed. If we then consider qualitative differences between normal and mutant lines, phenotypic respiratory differences become still more convincing. Respiratory stimulation or inhibition (expressed in percent) and respiratory quotient are dimensionless quantities which cannot be influenced by the basis of measurement. As shown in figure 2, the more rapid decline of RQ with age in mutant leaf tissue (especially dwarf-1) clearly differentiates these plants from the normal and the basis for this and other distinctions must lie within the respective respiratory machinery.

Summary

Using manometric methods, respiratory features were investigated in third leaf tissue of maize grown under standard conditions. Two mutants, dwarf-1 and brachytic-2, were compared with each other and with the normal. After the mutants had been introduced into the inbred line, progeny were repeatedly back crossed to increase homozygosity. These essentially isogenic maize lines had been the subject of detailed morphological studies by others and the comparison was thus extended to include certain physiological characteristics. Measurements included rates of oxygen uptake and both aerobic and anaerobic carbon dioxide production, response to succinate and glucose feeding, sensitivity to sodium azide and to 2,4-dinitrophenol, and the effect of pH of the bathing medium on certain of these effects. By some of these criteria plants of the three different maize lines were indistinguishable while in other ways clear cut differences were detected. Of greatest significance were those differences in unit-free characteristics such as RQ or percent rate stimulation in response to added substrate. The conclusion is reached that the mutant genes exert an influence on the respiratory mechanism giving rise to distinct though similar phenotypes.

The advice and the assistance of Professor E. C. Abbe and Dr. O. L. Stein are gratefully acknowledged.

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ASYMMETRIC DISTRIBUTION OF C^{14} IN THE GLUCOSE PHOSPHATES FORMED DURING PHOTOSYNTHESIS [1,2]

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While Bassham, Benson, Kay, Harris, Wilson and Calvin (1) have found a symmetrical distribution of C^{14} in fructose isolated from Scenedesmus following a brief photosynthesis in $C^{14}O_2$, Gibbs and Kandler (2) have recently reported an asymmetric labeling pattern in the glucose moiety of sucrose and starch both in Chlorella as well as the leaves of several higher plants. In our previous communication only the glucose of the end products of photosynthesis was investigated. In this note, the distribution of tracer in compounds more closely associated with the photosynthesis cycle will be presented, namely the several glucose phosphate esters. Labeled $C^{14}O_2$ was used in photosynthesis by dense Chlorella suspensions (1.5 ml packed cells per 100 ml water) under two conditions: (1) illumination of the algae for 5 minutes in a nitrogen atmosphere before the introduction of $C^{14}O_2$ for a period of 10 seconds and (2) incubation of the cells with the $C^{14}O_2$ in the dark for five minutes before photosynthesis occurred for one minute. The photosynthesis was terminated by pouring the Chlorella suspension into boiling 80 % ethanol. The 80 % ethanol extract and two 20 % ethanol washings were concentrated under vacuum to 1 ml. This residue was analyzed by two-dimensional paper chromatography (solvents: water saturated phenol and butanol–propionic acid). The areas of the chromatogram occupied by the sugar phosphates were eluted and the eluate was subjected to enzymatic hydrolysis by phosphatase. The resulting free sugars were then separated by two-dimensional paper chromatography using the same solvent system as for the original plant extract. Glucose was detected in four distinct spots on the chromatogram of the original plant extract (fig 1).

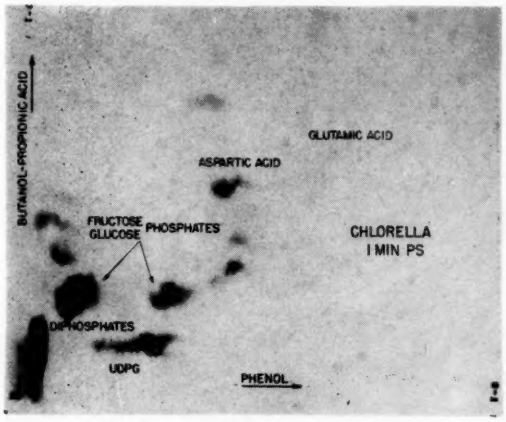

FIG. 1. Chromatogram of Chlorella extract (1-min photosynthesis) indicating the 4 glucose phosphate areas.

TABLE I

DISTRIBUTION OF C^{14} IN GLUCOSE PHOSPHATE ESTERS

EXPT NO.	AREA	TRACER CONTENT OF GLUCOSE CARBON ATOMS					
		1	2	3	4	5	6
		cpm/mg carbon					
1	Monophosphate	51	43	186	243	19	35
2	UDPG *	88	72	468	530	43	59
3	Unknown glucose phosphate	216	200	838	1074	117	141
4	Monophosphate	85	90	550	595	12	16
5	UDPG	83	80	730	811	12	20

In experiments 1, 2 and 3, the Chlorella was incubated with the $C^{14}O_2$ in the dark for 5 min before an illumination of 4000 ft-c for 1 min was begun.

In experiments 4 and 5, the Chlorella was illuminated 5 min in a nitrogen atmosphere before the introduction of $C^{14}O_2$. Photosynthesis time was 10 sec. Illumination intensity was 4000 ft-c

* UDPG = Uridinediphosphate glucose.

Analogous to the chromatograms published by Bassham, Shibata, Steenberg, Bourdon and Calvin (3), glucose was found in the diphosphate area (close to the origin and associated with fructose and ribulose), monophosphate area (again associated with fructose) and the uridine diphosphate glucose (UDPG) area. The fourth area finds no analogy on the chromatograms of the California group. It is located on all of our chromatograms between the diphosphate and the phosphoglyceric acid areas and contains tracer in a ratio of approximately 60 % glucose and 40 % fructose. The glucose of the various areas was degraded by the *Leuconostoc mesenteroides* method which permits a determination of the C^{14} content of the individual carbon atoms (4).

The data listed in table I shows that in each glucose phosphate ester, the specific activity of carbon atom 3 (C-3) was significantly less than that of C-4; however, C-1 (glucose aldehyde carbon) and C-2 were higher than that of C-5 and C-6. This asymmetric type of labeling in the glucose phosphate esters is

[1] Received August 3, 1956.

[2] Research carried out at the Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

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similar to that reported in our earlier communication for the glucose moiety of sucrose and starch.

If a lower activity in C-3 in relation to C-4 was caused by the presence of a pool of unlabeled dihydroxyacetone phosphate as suggested by the California group (1), then a corresponding dilution of C-1 and C-2 would be expected. The marked difference between the ratio of C-3 to C-1 and/or C-2 and C-4 to C-5 and/or C-6 indicates that it is unlikely that two equal types of triose phosphates are combined to yield a hexose phosphate.

Summary

Photosynthesis was carried out with Chlorella using labelled $C^{14}O_2$: 1) after illumination for 5 minutes in atmosphere of N_2 and for 10 seconds after introduction of $C^{14}O_2$ and 2) incubation of the cells with $C^{14}O_2$ in the dark for 5 minutes and then 1 minute of light. The glucose was isolated from the sugar phosphates, and the labelling in the carbon atoms followed. The labelling pattern is not that which is expected if two equal triosephosphates combined to yield an hexosephosphate.

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Endowed Chair of Conservation: The University of Louisville has announced the creation of an endowed professorship of conservation, made possible by a gift of $235,000 from anonymous donors. This chair in the Biology Department will be named in honor of Tom Wallace, editor emeritus of *The Louisville Times* and a national figure in the conservation movement of this country.

A substrate favorable for nurturing a conservation program already exists in this department, where courses and research in several aspects of field biology have received increasing emphasis in recent years.

President Philip Davidson commented: "This is a major development in the university's program and we are signally honored to have Mr. Wallace's name associated with the university in so important and significant a way. It gives us the greatest possible pleasure to join in honoring one of the really great citizens of our day in this manner, and we pledge our efforts to make this one of the most distinguished programs of conservation in the country."

Mr. Wallace has served as president of the Izaak Walton League, as a member of the advisory board of the National Parks Service and as chairman of the National Conference on State Parks. He founded the Ohio Valley Regional Conference on State Parks and is an honorary member of the Society of American Foresters and the American Society of Landscape Architects. Through editorials and columns in *The Louisville Times* over several decades he has made a national reputation as a foe of resource plunderers.

An appointment to this new position will be made in the near future.—William M. Clay, *Dept. of Biology, University of Louisville, Louisville 8, Kentncky.*

Wightman Wells Garner

Codiscoverer of Photoperiodism in Plants
1875–1956

Although among his colleagues and coworkers of the United States Department of Agriculture, Dr. W. W. Garner was best known as a national authority on tobacco culture, to plant physiologists throughout the world, his name with that of Dr. H. A. Allard, is associated with the monumental discovery and demonstration of photoperiodism in plants. Very few recent discoveries in botany have had as far reaching effects on studies and understanding of growth and reproduction. In both the theoretical and applied fields of plant research, photoperiodism is being used everywhere as a convenient means of obtaining plants of a desirable type of development. This despite the fact that the mechanism of photoperiodism, like that of many other important plant phenomena, is still unsolved. The early publications on photoperiodism by Garner and Allard are now classics of botanical literature.

Dr. W. W. Garner was born July 15, 1875, at Timmonsville, South Carolina. He received the B.A. degree from the University of South Carolina in 1896 and four years later the Ph.D. degree from the Johns Hopkins University. His first association with the United States Department of Agriculture was in the Bureau of Chemistry in 1904. A year later he was transferred to the newly organized Bureau of Plant Industry where his long and fruitful experimental work on the tobacco plant began. In 1908 Dr. Garner was appointed physiologist in charge of Tobacco and Plant Nutrition Investigations. Under his leadership and inspiration a large number of outstanding investigations were made in practically all phases of tobacco culture. Of particular interest to plant physiologists are the studies on mineral and nitrogen nutrition, the relation of soil nutrient to the formation and the concentration of nicotine in this plant and the perfection of a convenient method for the determination of this alkaloid. The most outstanding results of these prolonged investigations are summarized in a number of scientific papers and in a book, "The Production of Tobacco," which was first published in 1946, one year after Dr. Garner's retirement. This book is the recognized authority in this field.

In 1937 the Stephen Hales Prize for distinction in research, primarily the development of the concept of photoperiodism, was awarded to Dr. Garner by the American Society of Plant Physiologists. On this seventieth anniversary a portrait of him with a biographical note was printed in *Plant Physiology* (20: 464–466. 1945). For his accomplishments in pure and applied plant science he received in 1937 the honorary degrees of Doctor of Science from both Clemson College and the North Carolina State College.

Dr. Garner died March 19, 1956 in Washington, D. C.—A. E. Murneek.